311. *Carbohydrates of the Red Alga,* Porphyra umbilicalis.

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Extraction of the alga with hot water gives dialysable oligosaccharides and a polysaccharide fraction, the principal constituents of which are a galactan sulphate and floridean starch. The galactan sulphate, isolated by precipitation with cetylpyridinium chloride, contains D- and L-galactose, 6-*O*-methyl-D-galactose, and 3,6-anhydro-L-galactose.

The carbohydrates of red algæ (Rhodophyceæ) show a general similarity throughout the class in that they consist of (i) a water-soluble mucilage,[1] which usually contains a high proportion of a galactan, (ii) an iodophilic polysaccharide, floridean starch, which resembles glycogen or amylopectin in structure,[2] (iii) glycosides and cyclitols of low molecular weight,[3] and (iv) structural polysaccharides of the cell wall.[4] We are concerned in this paper with the carbohydrates present in the red alga *Porphyra umbilicalis* (L.) Kütz, f. *umbilicalis*, which occurs widely around the shores of Britain and is eaten in Wales under the name " laver-bread."

The dried weed, which was collected in August on the North Wales Coast, was extracted successively with cold and hot water, dilute acid, dilute alkali, and finally with hot concentrated alkali. Complete acidic hydrolysis of each extract and chromatographic examination of the sugars produced gave results essentially similar to those reported by other workers.[4,5] Since cold and hot water extracted similar material, and also since dilute acid extracted only inorganic material, extractions with cold water and with dilute acid were omitted in subsequent extractions.

More than half the dry weight of the alga was soluble in hot water and of this *ca.* 10% consisted of dialysable oligosaccharides of low molecular weight. Paper chromatography indicated that these oligosaccharides were non-reducing and that hydrolysis gave glycerol and galactose as main components, together with smaller amounts of glucose and mannose.

[1] Hirst, *Proc. Chem. Soc.*, 1958, 177; Mori, *Adv. Carbohydrate Chem.*, 1953, **8**, 315.
[2] Fleming, Hirst, and Manners, *J.*, 1956, 2831; Peat, Turvey, and Evans, *J.*, 1959, 3223, 3341.
[3] Lindberg, *Acta Chem. Scand.*, 1955, **9**, 1097.
[4] Cronshaw, Myers, and Preston, *Biochim. Biophys. Acta*, 1958, **27**, 89.
[5] Jones, *J.*, 1950, 3292.

This is consistent with the presence in the alga of oligosaccharides of the floridoside type.[3] Floridoside and isofloridoside have since been isolated and characterised from the seaweed.[6] No oligosaccharides were present in other than the hot-water fractions. The polysaccharide fractions extracted by cold N-sodium hydroxide and by hot 20% sodium hydroxide both gave mannose, xylose, and glucose on hydrolysis, but they differed in that mannose was the main constituent from the dilute alkaline extracts while xylose was more abundant in the extracts with concentrated alkali. This is in contrast to the results obtained by previous workers.[4,5] It is apparent that mannan and xylan, together with some glucan, are the skeletal polysaccharides of this alga.

The water-soluble polysaccharides presented a complex pattern, the main constituent sugars being galactose and a sugar with the R_F value of fucose but subsequently identified as 6-*O*-methyl-D-galactose; others were glucose, mannose, arabinose, and xylose. The hydrolysate also contained degradation products of the furfuraldehyde type, later shown to be derived from 3,6-anhydro-L-galactose. No ribose or uronic acids were detected although these have been reported to be present in this alga.[4] The extract contained ester sulphate and also gave a weak red-brown stain with dilute iodine solution, indicating the presence of floridean starch. During many attempts at fractionation of this extract, galactose, 6-*O*-methylgalactose, and ester sulphate always occurred together and, therefore, presumably arise from the same polysaccharide (galactan), which also contains residues of 3,6-anhydro-L-galactose.

Fractionation of the Water-soluble Polysaccharides.—We wished to obtain specimens of the galactan and, if possible, of the floridean starch, both of which occurred in the fraction soluble in hot water. After many attempts at fractionation, the use of cationic detergents [7] as precipitants gave some success. At neutral pH, cetylpyridinium chloride precipitated the galactan component in good yield and with only traces of contaminating polysaccharides (fraction A1; see Table). When the pH of the supernatant solution was adjusted to 13,[8] a further precipitate (fraction A2) was obtained and this contained most of the floridean starch, together with some galactan and other polysaccharides. Fraction

Properties of fractions obtained from water extract of alga.

	Fraction			
	A1	A2(i)	A2(ii)	A3
Yield from fraction A (%)	53	1·2	2·4	1·0
SO_4^{2-} (%)	13·3	3·3		9·7
Iodine stain	—	—	+	—
Content of sugars (%): *				
Galactose	37·2	21·0	23·5	12·3
6-*O*-Methyl D-galactose	22·7	8·2	2·8	3·8
3,6-Anhydrogalactose	11·7		1·3	2·4
Glucose	0·2	3·7	42·0	22·0
Mannose	0·2	0·5	1·0	1·0
Xylose	0·5	1·5	1·1	6·7

* Determined after complete hydrolysis. Very small amounts of arabinose were also present in fractions A2(i) and A3.

A2 was further fractionated by using " Cetavlon " (cetyltrimethylammonium bromide) at pH 13 as precipitant: the floridean starch was not precipitated thereby [fraction A2(ii); Table] and some of the galactan impurity was removed in the precipitate [fraction A2(i); Table]. This subfractionation was not always reproducible but depended on the batch of " Cetavlon " used as precipitant. Since this detergent is a commercial preparation, it is almost certainly polydisperse with respect to alkyl chain-length. Scott [9] has

[6] Peat and Rees, *Biochem. J.*, in the press.
[7] Bera, Foster, and Stacey, *J.*, 1955, 3788; Scott, *Biochim. Biophys. Acta*, 1955, **18**, 428.
[8] Barker, Stacey, and Zweifel, *Chem. and Ind.*, 1957, 330; Palmstierna, Scott, and Gardell, *Acta Chem. Scand.*, 1957, **11**, 1792.
[9] Scott, " Methods of Biochemical Analysis," ed. Glick, Interscience Publ. Inc., New York, 1960, Vol. VIII.

recently shown the importance of the chain-length in determining the efficiency of a cationic detergent as a precipitant for acidic polysaccharides. It is apparent that different batches of the " Cetavlon " varied somewhat in composition. A similar subfractionation of fraction A2 was, however, obtained on a small scale by electrophoresis on thick filter paper, two fractions essentially similar to A2(i) and A2(ii) being obtained.

For a detailed study of the galactan we have used fraction A1 obtained with cetylpyridinium chloride. This fraction contained N 0·3% and ash 20·5% and had $[\alpha]_D^{19}$ −79° (in water). It migrated as a single zone on paper electrophoresis in neutral buffer and could not be separated into further fractions by using potassium chloride as gelling agent.[10] To characterise the constituent sugars, mercaptolysis[11] was adopted. Mercaptolysis, followed by removal of thioacetal groups, gave a sugar syrup which was fractionated by chromatography on charcoal. The charcoal used was " Ultrasorb S.C. 120/240 " since this adsorbent retains monosaccharides, while salts are eluted with water, and it also gives a better separation of monosaccharides than less active charcoals.[12] Galactose, obtained from this column, was freed from traces of mannose by preparative paper chromatography. Its optical rotation and also the optical rotation of the derived galactosazone indicated a ratio of D- to L-galactose of 1 : 1·1. The 6-*O*-methyl-D-galactose crystallised and was compared with a specimen synthesized by Freudenberg and Smeykal's method.[13] From its optical rotation and that of its osazone, it is apparent that only the D-form occurs in this alga. Little 3,6-anhydro-L-galactose was obtained by this method and an alternative method of isolation was devised, based on that of Clingman *et al.*[14] Cautious hydrolysis of the polysaccharide with 0·5N-sulphuric acid resulted in scission of the 3,6-anhydrogalactosidic linkages without causing marked degradation of this sugar. Subsequent reduction with sodium borohydride and more vigorous hydrolysis then gave a mixture containing the relatively stable L-3,6-anhydrogalactitol (D-1,4-anhydrogalactitol), which was separated by column chromatography and characterised. The presence of 6-*O*-methyl-D-galactose deserves some comment since it has been reported from only a few, botanically related species of algæ, *P. capensis*,[15] *P. crispata*,[16] and *Bangia fuscopurpurea*.[17] It may well be a distinguishing feature of certain classes of alga.

Quantitative analysis of the sugars constituting the galactan gave the molar ratio galactose : 6-*O*-methyl-D-galactose : 3,6-anhydro-L-galactose : ester sulphate as 100 : 57 : 35 : 65. It is apparent, therefore, that this polysaccharide resembles that isolated from *P. capensis*[15] in the ester-sulphate content and in the component sugars. The main difference between these two polysaccharides is in the content of 3,6-anhydro-L-galactose, which appears to be lower in the *P. umbilicalis* polysaccharide.

Experimental

General Methods.—Paper chromatography and electrophoresis. The methods used for paper chromatography have been described,[18] but for the detection of sugar zones, in addition to the reagents described,[18] the *p*-anisidine hydrochloride[19] and periodate–benzidine reagents[20] were used, the former because it gives different colours with different classes of sugar and the latter for detection of glycerol and other non-reducing sugars. For the electrophoresis of polysaccharides, an acetic acid–pyridine buffer[21] was used with Whatman 3 MM. paper. Acidic

[10] Smith, Cook, and Neal, *Arch. Biochem. Biophys.*, 1954, **53**, 192.
[11] Araki and Hirase, *Bull. Chem. Soc. Japan*, 1953, **26**, 463; Percival, *Chem. and Ind.*, 1954, 1487.
[12] Hughes and Whelan, *Chem. and Ind.*, 1958, 884.
[13] Freudenberg and Smeykal, *Ber.*, 1926, **59**, 100.
[14] Clingman, Nunn, and Stephen, *J.*, 1957, 197.
[15] Nunn and von Holdt, *J.*, 1957, 1094.
[16] Jong-Ching Su, personal communication.
[17] Ya Chen Wu and Hon-Kai Ho, *J. Chinese Chem. Soc.* (*Formosa*), 1959, **6**, 84.
[18] Peat, Whelan, and Roberts, *J.*, 1957, 3916.
[19] Hough, Jones, and Wadman, *J.*, 1950, 1702.
[20] Cifonelli and Smith, *Analyt. Chem.*, 1954, **26**, 1132.
[21] Peat, Turvey, Clancy, and Williams, *J.*, 1960, 4761.

polysaccharides were detected with Toluidine Blue,[22] and the floridean starch with dilute iodine solution.

Hydrolysis of polysaccharides. The polysaccharide (1—20 mg.) was heated with 1·5N-sulphuric acid (0·5—2 ml.) at 100° for 3 hr., cooled, and neutralised with barium carbonate. After being filtered, the solution was concentrated at 35—40° and portions were examined by paper chromatography. If the polysaccharide was not soluble in the dilute acid, it was heated in a sealed tube with 90% formic acid (2 ml.) at 100° for 5 hr.; the cooled solution was diluted with water (10 vol.) and evaporated to dryness and the residue hydrolysed with N-sulphuric acid (5—10 ml.) at 100° for 5 hr., then neutralised and examined as above.

Analytical.—Quantitative analysis of a sugar mixture. Pridham's method [23] was used except that sugar solutions were applied to the filter paper with a modified micropipette.[24]

Estimation of 3,6-anhydrogalactose in a polysaccharide. The method, modified from that of O'Neill,[25] depends on the acidic degradation of 3,6-anhydrogalactose to 5-hydroxymethylfurfuraldehyde, which is estimated spectrophotometrically. The polysaccharide (40—100 mg.) was dissolved in water (20 ml.), 3N-hydrochloric acid (1·25 ml.) was added, and the solution diluted to 25 ml. Portions (2 ml. each) of this solution were sealed in tubes and heated at 100° for varying times (2—24 hr.). Each tube was then cooled, and the contents were washed into a 100 ml. flask containing barium carbonate (1 g.). The flasks were heated at 100° for 5 min., and water was added to 100 ml. The resulting suspension was filtered and the optical density of the filtrate measured at 285 mμ in 1 cm. cells. A curve of the logarithm of the optical density against time of heating was constructed and the later, linear portion of the curve extrapolated to zero time. From the known molar extinction coefficient of 5-hydroxymethylfurfuraldehyde (16,500) the concentration was determined.

Total sulphate. The material (50—200 mg.) was oxidised with concentrated nitric acid (20 ml.) containing sodium chloride (5—10 mg.). When the nitric acid had been evaporated off, a further 20 ml. was added and evaporated off. Last traces of nitric acid were removed by addition of concentrated hydrochloric acid and evaporation to dryness. The residue was dissolved in water and the liberated sulphate determined gravimetrically as barium sulphate.

Extraction of Seaweed.—(a) *With water.* The fresh seaweed was lightly washed with water and dried at 35° in air before being ground to a fine powder. The powder (100 g.) was stirred in water (1·5 l.) at 100° for 16 hr., and, after cooling, the residue was removed by centrifugation. This residue was then extracted as above with further portions (1·5 l. each) of water until no further material was extracted. The combined extracts were evaporated (35—40°) to 2 l. and poured slowly, with stirring, into ethanol (3 vol.). The fibrous precipitate was collected on the centrifuge, redissolved in water, dialysed against distilled water (3 × 5 l.), and reprecipitated with ethanol (3 vol.). The fibrous product (fraction A) was washed with ethanol and ether and dried over phosphoric oxide (yield 41·7 g.; ash 12·2%; SO_4^{2-}, 10·2%). After hydrolysis the following sugars were present: galactose (3+), 6-*O*-methylgalactose (3+), glucose (2+), xylose (+), mannose (+), arabinose (+), and 5-hydroxymethylfurfuraldehyde (2+).

The supernatant solution, obtained after precipitation of fraction A from the extracts, was evaporated at 35—40° to a syrup (27 g.; ash 80%), which was extracted four times with portions (100 ml. each) of boiling 85% methanol. The combined extracts were decolorised with charcoal and evaporated to a syrup (11·8 g.). A portion of this syrup was examined by paper chromatography, the periodate–benzidine spray being used to locate non-reducing sugar zones. Two main non-reducing components were detected together with traces of reducing oligosaccharides. A further portion on hydrolysis gave the following sugars: galactose (3+), glycerol (3+), glucose (+), and mannose (+).

(b) *With cold, dilute alkali.* The residue from the previous extraction was stirred at room temperature with N-sodium hydroxide (1 l.) for 16 hr. and the solids were removed by centrifugation. The solids were re-extracted repeatedly with the reagent until no further material was extracted (10 times). The combined green extracts, on acidification with hydrochloric acid (pH 3), gave a precipitate (fraction B1), which was separated, washed with alcohol to remove colouring matter, and dried as before. The residual solution was evaporated to 1 l. at 35—40° and pH 6, dialysed against tap water for 3 days, and poured into ethanol (3 vol.), to give a

[22] Ricketts, Walton, and Saddington, *Biochem. J.*, 1954, **58**, 532.
[23] Pridham, *Analyt. Chem.*, 1956, **28**, 1967.
[24] Harkins and Anderson, *J. Amer. Chem. Soc.*, 1937, **59**, 2189.
[25] O'Neill, *J. Amer. Chem. Soc.*, 1955, **77**, 2837.

precipitate (fraction B2), which was recovered as for fraction B1. The supernatant solution contained no detectable carbohydrate. On hydrolysis fraction B1 (4·4 g.) gave mannose (3+), xylose (+), galactose (+), and glucose (trace); fraction B2 (2·0 g.) gave mannose (2+), glucose (2+), galactose (+), and xylose (trace).

(c) *With hot, concentrated alkali.* The residue from the previous extraction was stirred with 20% (w/v) sodium hydroxide (500 ml.) at 100° for 12 hr.; the solids were removed and re-extracted 3 times. The final residue (11·7 g.; ash 98%) was rejected. The extracts were acidified with hydrochloric acid to pH 3 and the precipitate formed was recovered as for previous fractions (fraction C1). The supernatant solution was dialysed until free from chloride ions, concentrated to 500 ml., and poured into ethanol (3 vol.). The precipitate was recovered as before (fraction C2). On hydrolysis, fraction C1 (5·1 g.) gave xylose (3+) and glucose (trace); fraction C2 (3·6 g.) gave xylose (2+), glucose (+), and mannose (+).

Sub-fractionation of Fraction A *with Detergents.*—Fraction A (165 g.) was stirred with water (9 l.) at 60° for 4 hr. and the residue (32 g.) then removed on the centrifuge. To the supernatant solution, cetylpyridinium chloride (120 g. per l.) was added until the precipitate just flocculated. The precipitated complex was collected on the centrifuge, the supernatant solution (X) being retained, and the precipitate was washed with 2% sodium chloride solution. The complex was redissolved by stirring overnight in 10% aqueous sodium chloride (5 l.), the insoluble residues were discarded, and the complex was reprecipitated by dialysing the solution against tap water. The complex was recovered on the centrifuge and redissolved in 10% aqueous sodium chloride (5 l.), and the polysaccharide was precipitated by the addition of ethanol (2 vol.). The precipitated polysaccharide was redissolved in water (8 l.), dialysed against tap water, and reprecipitated by addition of ethanol (3 vol.). The polysaccharide (fraction A1, Table) was washed with alcohol and ether, and dried over phosphoric oxide. To the solution (X) remaining after removal of the precipitated complex, sodium hydroxide was added to a concentration of 0·1N (pH 13) and the precipitate was collected on the centrifuge. This precipitate was washed with 0·1N-sodium hydroxide and redissolved in 10% sodium chloride solution, and the polysaccharide was recovered by pouring the mixture into ethanol (4 vol.), redissolved in water, dialysed, reprecipitated, and dried as above (fraction A2). The solution remaining after precipitation of the complex at pH 13 was neutralised and treated with potassium iodide to remove excess of detergent.[26] It was then dialysed against tap water for 2 days and poured into ethanol (3 vol.). The precipitated polysaccharides were washed and dried as above (fraction A3, Table).

Resolution of Fraction A2.—(a) *With "Cetavlon."* Fraction A2 (100 mg.) was dissolved in 0·1N-sodium hydroxide, and a solution of "Cetavlon" (B.D.H., 10 g. per 100 ml.) was added until the precipitate just flocculated. The precipitated complex was collected and dissolved in 2N-acetic acid, and the solution was poured into ethanol (4 vol.). The precipitate was redissolved, precipitated, and dried as above [fraction A2(i), Table]. The solution remaining after precipitation of the "Cetavlon" complex was neutralised, freed from "Cetavlon" by potassium iodide,[26] and dialysed against tap water. The polysaccharides were recovered from this solution by precipitation with ethanol (4 vol.), washed, and dried as above [fraction A2(ii), Table].

(b) *By paper electrophoresis.* Fraction A2 (20 mg.) was applied to Whatman 3 MM. paper and subjected to electrophoresis in neutral buffer. The paper was then dried in air and guide-strips treated with Toluidine Blue reagent to detect migrated zones of acidic polysaccharides. The remaining paper was lightly sprayed with dilute iodine solution to locate zones of floridean starch. The respective zones were cut from the paper, and polysaccharides recovered in the usual way. The sugars obtained on hydrolysis indicated that the migrating and the non-migrating (floridean starch) zone corresponded in composition to fractions A2(i) and A2(ii), respectively.

Mercaptolysis of Fraction A1.—The fraction (5 g.) was treated with hydrochloric acid and ethanethiol by O'Neill's method,[25] to give a mixture (4 g.) of sugar thioacetals. The mixture was stirred overnight at 50° with water (500 ml.) containing mercuric chloride (9 g.) and cadmium carbonate (15 g.). The mixture was then filtered and the solution saturated with hydrogen sulphide, filtered, freed from hydrogen sulphide by aeration, and neutralised with sodium hydroxide. The solution was concentrated at 30° to 100 ml. and adsorbed on a column (45 × 6 cm.) of charcoal–Celite (1 : 1, by weight), which was eluted with a linear gradient of

[26] Scott, *Chem. and Ind.*, 1955, 168.

ethanol in water (0—6% in 25 l.) and finally with 10% aqueous ethyl methyl ketone. The charcoal used was " Ultrasorb S.C. 120/240 " (British Carbo-Norit Union, Ltd.). Fractions (200 ml. each) were collected and tested for carbohydrates with the anthrone reagent.[27] The first material to be eluted was a small amount of xylose and this was followed by two major fractions containing galactose and 6-*O*-methylgalactose. The ethyl methyl ketone-eluted material giving a positive Seliwanoff reaction, but only 13 mg. of 3,6-anhydrogalactose were present.

Identification of DL-*galactose.* The galactose (0·284 g.) from the column was contaminated with a trace of mannose and it was therefore purified by chromatography on thick filter paper. The syrup obtained was dissolved in water and treated with Somogyi's deproteinising reagent,[28] and its optical rotation was measured, the concentration of galactose also being determined by Somogyi's method.[28] The value, −3·9°, obtained for the specific rotation indicated a D- to L-galactose ratio of 1 : 1·1. The galactose was characterised by conversion of a portion (140 mg.) into mucic acid, m. p. 205°, and of another portion (100 mg.) into galactosazone, m. p. 194°, $[\alpha]_D^{19}$ −5·4° (*c* 0·65 in pyridine). This value for the specific rotation of the galactosazone also indicates a D- to L-galactose ratio of 1 : 1·1.

Identification of 6-O-*methyl*-D-*galactose.* The second peak from the column was evaporated to a syrup, which contained 176 mg. of 6-*O*-methylgalactose as determined by Somogyi's method after deproteinisation.[28] The syrup was extracted with ethanol (3 × 50 ml.) and the extracts were evaporated to 20 ml. and seeded. The crystalline product had $[\alpha]_D^{18}$ +74° (final value in water), m. p. 115—116°, undepressed on admixture with 6-*O*-methyl-D-galactose synthesized by Freudenberg and Smeykal's method.[13] The osazone had $[\alpha]_D^{18}$ +137° (0 hr.) ⟶ +95° (24 hr.) in dry pyridine and m. p. 196·5—197°, mixed m. p. with authentic 6-*O*-methyl-D-galactosazone 197—197·5°.

Identification of 3,6-*Anhydro*-L-*galactose.*—Fraction A1 (18 g.) was stirred at 50° in 0·5N-sulphuric acid (2 l.) for 20 hr. and then cooled. After being neutralised with barium carbonate, the solution was filtered and evaporated to 150 ml. Sodium borohydride (2 g.) was then added and the mixture agitated gently for 16 hr., by which time only a weak Seliwanoff test was given by the solution. Excess of the borohydride was destroyed by 3N-sulphuric acid, and then sufficient acid was added to give a N-solution, which was heated at 100° for 20 hr., neutralised with barium carbonate, filtered, and evaporated to 50 ml. This solution was chromatographed on a column (120 × 5 cm.) of charcoal–Celite (B.D.H. " activated charcoal "), which was eluted with a gradient of 0—5% ethyl methyl ketone. Fractions were examined by paper chromatography and those fractions containing 3,6-anhydrogalactitol as the main constituent were bulked and evaporated to a syrup. The syrup was fractionated by chromatography on thick filter paper, 3,6-anhydro-L-galactitol being obtained as a syrup (0·57 g.), $[\alpha]_D^{19}$ −16·6° (in water). The fully benzoylated derivative [29] had m. p. 102·5—103·5°, $[\alpha]_D^{19}$ +40·5°.

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[27] Loewus, *Analyt. Chem.*, 1952, **24**, 219.
[28] Somogyi, *J. Biol. Chem.*, 1954, **160**, 61, 69.
[29] Ness, Fletcher, and Hudson, *J. Amer. Chem. Soc.*, 1951, **73**, 3742.