## 1104. *Porphyran: A Polysaccharide with a Masked Repeating Structure* *

By N. S. Anderson and D. A. Rees

Within the limits of the available methods for structure analysis, porphyran has a linear structure consisting of 3-linked β-D-galactosyl and 4-linked α-L-galactosyl units in alternating sequence. The regularity of this repeating arrangement is masked by (i) the partial occurrence of the D-galactosyl unit as the 6-*O*-methyl ether, and (ii) the occurrence of the L-galactosyl unit as both the 6-sulphate and the 3,6-anhydride.

Porphyran is obtained in copious amounts by hot-water extraction of red seaweeds of the genus *Porphyra*.[1,2] The constitution appears complex at first sight because sugar units of four types are present, namely D-galactose, L-galactose, 6-*O*-methyl-D-galactose, and 3,6-anhydro-L-galactose, together with ester sulphate, and the polysaccharide has not so far been separated into distinct fractions. The proportions of the components vary widely from sample to sample but certain regularities are apparent, notably that virtually all the L-galactose is sulphated at position 6,[3-5] that this always accounts for nearly all the ester

* A brief account of part of this work has appeared, N. S. Anderson, T. C. S. Dolan, and D. A. Rees, *Nature*, 1965, **205**, 1060.

[1] J. R. Nunn and M. M. von Holdt, *J.*, 1957, 1094.
[2] S. Peat, J. R. Turvey, and D. A. Rees, *J.*, 1961, 1590.
[3] J. R. Turvey and D. A. Rees, *Nature*, 1961, **189**, 831.
[4] D. A. Rees, *J.*, 1961, 5168.
[5] D. A. Rees and E. Conway, *Biochem. J.*, 1962, **84**, 411.

sulphate in the polysaccharide, and that the sum of the proportions of L-galactose 6-sulphate and 3,6-anhydro-L-galactose is always equal to the sum of the proportions of D-galactose and 6-*O*-methyl-D-galactose. It is believed from the results of periodate oxidation,[4] alkaline elimination,[4] and partial hydrolysis,[6] and also from studies of the biosynthesis of porphyran [7] that the D-galactose and 6-*O*-methyl-D-galactose derivatives are each linked through position 3, and that the L-galactose 6-sulphate and 3,6-anhydro-L-galactose are each linked through position 4. Suggestions regarding the distribution of linkages have also been made, and these are mentioned later in this Paper.

We now report the characterisation of the glycosidic linkages in porphyran by a combination of methylation analysis and partial fragmentation, and give evidence for a type of repeating structure. The L-galactose 6-sulphate units in porphyran were converted into 3,6-anhydrogalactose by alkaline elimination, and the product was completely methylated. Hydrolysis followed by paper and gas–liquid chromatography (g.l.c.) showed the presence of 2,4,6-tri-*O*-methylgalactose and 3,6-anhydro-2-*O*-methylgalactose as the only products present in sufficiently large concentrations to be of clear structural significance. It would therefore seem that the two types of D-units and the two types of L-units are structurally equivalent, and that there is no possibility of a branched structure. Some possible arrangements of the units in methylated alkali-modified porphyran are shown in formulæ (I)—(IV). Structure (IV) can be distinguished experimentally from the others, because 3,6-anhydrogalactosyl linkages are very much more labile to acid-catalysed cleavage than is usual for hexosyl linkages,[8,9] and even though completely selective hydrolysis is unlikely to be achieved, (IV) would give equimolar amounts of the two different monosaccharide units whereas the other structures (I)—(III) would give a greater proportion of 3,6-anhydro-2-*O*-methyl-L-galactose; moreover the alternating structure (IV) would give a relatively high yield of disaccharide.

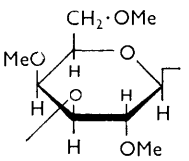
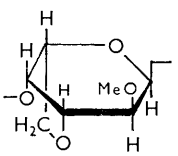
Methanolysis [10] followed by chromatography on a charcoal column gave various derivatives of " tetramethylagarobiose," a disaccharide that has previously been given the structure 3,6-anhydro-2-*O*-methyl-4-*O*-(2,4,6-tri-*O*-methyl-β-D-galactopyranosyl)-L-galactose (V).[10] The derivatives were the methyl β-glycoside (VIII), the dimethylacetal

-D-D-L-L-D-D-L-L-D-D-L-L-D-D-L-L-D-D-L-L-D-D-L-L- (I)

-D-D-D-D-D-D-D-D-D-D-D-D-L-L-L-L-L-L-L-L-L-L-L-L- (II)

-D-D-D-L-L-D-L-L-L-D-D-L-D-D-D-L-L-D-L-L-L- (III)

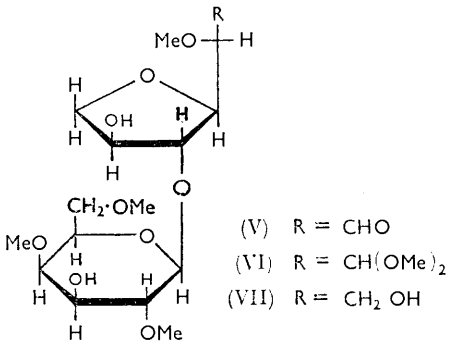
-D-L-D-L-D-L-D-L-D-L-D-L-D-L-D-L-D-L-D-L-D-D (IV)

-D- =  ; -L- = 

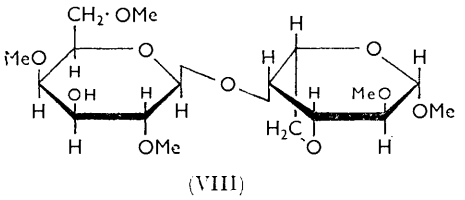
(VI), and the reducing sugar (V). The appearance of the sugar (V) was probably due to aqueous hydrolysis of the very acid-sensitive glycosides and/or dimethylacetal when the mixture of products was dissolved in water for the column separation. A trace of hydrogen chloride might have remained by inadvertent incomplete neutralisation of the methanolysate and provided the necessary acid catalysis.

[6] J. R. Turvey and T. P. Williams, in " Proceedings of the 4th International Seaweed Symposium (Biarritz, 1961)," Pergamon, Oxford, 1963.
[7] D. A. Rees, *Biochem. J.*, 1961, **81**, 347.
[8] W. N. Haworth, J. Jackson, and F. Smith *J.*, 1940, 620.
[9] C. Araki, in " Proceedings of the IVth International Congress of Biochemistry (Vienna, 1958)," Pergamon, Oxford, vol. I, p. 15.
[10] C. Araki and S. Hirase, *Bull. Chem. Soc. Japan*, 1960, **33**, 597.

The isolation of the agarobiose derivatives (V), (VI), (VIII) in 62% yield (based on the theoretical), shows the positions of both the linkages in the polysaccharide, and also that the configuration of the 1,4-linkage is the same as in agarose. We have confirmed the β-configuration[9] of this linkage by proton magnetic resonance (p.m.r.) spectroscopy of the tetramethyl-



(V) R = CHO
(VI) R = $CH(OMe)_2$
(VII) R = $CH_2OH$



(VIII)

agarobi-itol (VII) in deuterochloroform. On the basis of the general rule[11] that in the spectra of glycosides and methylated glycosides the signal from the anomeric proton appears at lower field than the signal from any other sugar-ring proton, a doublet at 5·57 τ was assigned to the anomeric proton. This assignment can only be tentative because the remainder of the spectrum was too complex to allow complete interpretation. The coupling constant ($J_{1,2} = 7{\cdot}3$ c./sec.) indicated the β-configuration. The α-configuration of the 1 ⟶ 3-linkage is deduced from the fact that the optical rotation of the methylated alkali-modified porphyran (−79°) resembles that of methylated agarose (−93°).[12,13]

No evidence was found for oligosaccharides of the types to be expected from structures (I)—(III). The mixture of monosaccharide products was not accurately analysed, but the yield of 3,6-anhydro-2-*O*-methyl-L-galactose derivatives certainly did not exceed the yield of 2,4,6-tri-*O*-methyl-D-galactose derivatives. This pattern of products is to be expected from the cleavage of a simple repeating structure (IV) rather than any of the alternatives.

In order to provide a further test of structure (IV), the total yield of agarobiose derivatives in the methanolysate was determined by g.l.c. and compared with the yield obtained when the methyl glycoside (VIII) was methanolysed similarly. The total amount of disaccharide derivatives present was found to diminish by only 8·5% during methanolysis of the glycoside. Correction of the total yield from the polysaccharide on the basis of this figure gave a " repeating-unit content " of 82%. Further correction on the basis of the accurately known sulphate ester content of methylated alkali-modified porphyran gave a value of 90% for the repeating unit content of the non-sulphated parts of the molecule. It is not possible to apply a reliable correction for the degree of undermethylation, but it can be calculated that a perfectly alternating polysaccharide which was methylated to within 97·5% of completion would have an apparent repeating-unit content as low as 90%.

We therefore conclude that if there is any deviation from the alternating pattern (IV) it is not within the power of our methods to detect it. Porphyran itself therefore consists of a linear chain of alternately 3-linked β-D-galactosyl and 4-linked α-L-galactosyl units, as has been suggested on the basis of analysis[5] and partial hydrolysis.[6] There was some loss of polysaccharide during the methylation step (the overall yield of the major fraction after alkali-modification and methylation was 59% with a further 16% in a minor fraction that was not examined in detail but which appeared to be similar to the major one), and this conclusion therefore involves the assumption that the porphyran molecules which

[11] R. J. Ferrier and N. F. Williams, *Chem. and Ind.*, 1964, 1696.
[12] C. Araki, personal communication.
[13] C. Araki and K. Arai, *Bull. Chem. Soc. Japan*, 1956, **29**, 339.

survived methylation were representative of the original population. The simple alternating sequence is masked because some of the D-galactose units are 6-*O*-methylated, the L-galactose occurs in the form of either of two derivatives (6-sulphate or 3,6-anhydride), and because of the small proportion of additional ester sulphate which has not yet been located in the molecule. The detection by paper chromatography of traces of 2,6- and 2,4-di-*O*-methylgalactoses in hydrolysates of the methylated polysaccharide suggests that this sulphate might occupy position 4 and/or position 6 of 1,3-linked galactose units, but the amounts of these sugars were so small that it is uncertain to what degree they were due to undermethylation.

## Experimental

*Porphyran.*—The material used in these experiments was extracted with boiling water (2 × 10 l. for 2-hr. periods) from fresh, wet *Porphyra umbilicalis* (6 lb.) collected on 24 November, 1963, near Port Seton, East Lothian. The combined liquid extracts were evaporated under diminished pressure (41), dialysed against running tap water and clarified on the centrifuge, and then poured into ethanol (20 l.). A little sodium hydroxide was added to aid coagulation. The precipitate was collected on a cloth filter, washed thoroughly with ethanol and then with ether and finally dried *in vacuo* (36 g.). Complete acid hydrolysis, followed by paper chromatography, showed the presence of galactose and 6-*O*-methylgalactose as the only sugars. Analysis by the methods of Rees and Conway [5] showed galactose ($C_6H_{10}O_5$), 28·7%; 6-*O*-methylgalactose ($C_7H_{12}O_5$) 20·5%; 3,6-anhydrogalactose ($C_6H_8O_4$) 14·2%: total sulphate ($SO_3$) 9·1%; and alkali-labile sulphate ($SO_3$) 8·7%. This corresponds to a molecule in which 25% of the units are 3,6-anhydrogalactose, 27% are L-galactose 6-sulphate, 18% are D-galactose, and 30% are 6-*O*-methyl-D-galactose.

*Qualitative Chromatographic methods.*—A search was first made for micro-chromatographic methods which would be suitable for the semi-quantitative analysis of mixtures of methylated derivatives of galactose, 3,6-anhydrogalactose, and agarobiose. Thin-layer chromatography on silica gel G coated on microscope slides (N. G. Richardson, unpublished method) was an excellent method for the analysis of simple mixtures (solvent; butane 2-one: spray; anisaldehyde–sulphuric acid [14]), for example for the preliminary examination of some of the column fractions (see below), but did not have sufficient resolving power for general use. G.l.c. [15] was more generally applicable, although there was no single set of conditions that would give all the necessary information. Methylated monosaccharide derivatives were separated using the butanediolsuccinate polyester column at 175°, and disaccharide derivatives were separated as their acetates on an SE-52 (3% on Gas-Chrom P) column at 200°. The acetates were prepared by heating the sugar (1 mg.) with pyridine (0·03 ml.) and acetic anhydride (0·015 ml.) in a sealed tube at 100° for 1 hr.; samples of the resulting solution (0·1 ml.) were injected on to the column without removal of the acetic anhydride or pyridine. All g.l.c. was carried out with a Pye Argon Chromatograph, fitted with a $^{90}Sr$ detector. For paper chromatography the solvents used were (A) butanol–ethanol–water (4 : 1 : 5, upper phase—for general purposes) and (B) ammonia (*d* 0·880)–water–butan-2-one (1 : 17 : 200, for the separation of isomeric methylated sugars); sprays were *p*-anisidine hydrochloride [16] for reducing sugars and periodate–Schiff [17] for non-reducing carbohydrates.

*Quantitative G.l.c. of Carbohydrate Derivatives.*—A Pye Argon Chromatograph with a $^{90}Sr$ detector and closed-liquid injection system was used, with 1,2:3,4-di-*O*-isopropylidene-6-*O*-toluene-*p*-sulphonyl-α-D-galactopyranose as the internal standard. The mixture was heated for 1 hr. at 100° with acetic anhydride (100 parts) and pyridine (200 parts). After evaporation to dryness and distillation of methanol from the residue, the material was dissolved in chloroform for analysis. Results were reproducible to at least ±1·5% whether peak heights or areas (electronic integrator) were used. The detector (linear response) was calibrated on the same day as each analysis.

*Alkaline Elimination of 6-Sulphate from Porphyran.*—This followed Rees' method.[4] Porphyran (15 g.) was dissolved in water (2·3 l.) and reduced at room temperature with potassium borohydride (1·9 g.; 3 days). More borohydride (28 g.) was added together with sodium

[14] E. Stahl and U. Kaltenbach, *J. Chromatog.*, 1961, **5**, 351.
[15] G. O. Aspinall, *J.*, 1963, 1676.
[16] L. Hough, J. K. N. Jones, and W. H. Wadman, *J.*, 1950, 1702.
[17] F. E. Hardy and J. G. Buchanan, *J.*, 1963, 5881.

hydroxide (93 g.) and the temperature was raised to 80° on a water-bath. When analysis showed that the concentration of combined 3,6-anhydrogalactose present in the solution had reached a constant value (180 min.), the solution was cooled to room temperature.

*Methylation of Alkali-modified Porphyran.*—This was carried out on the above solution without isolating the modified polysaccharide. The solution was cooled in ice, and then sodium hydroxide (707 g.) was added with vigorous stirring in an atmosphere of nitrogen followed by dimethyl-sulphate (300 ml.), the latter reagent being added slowly over 4 hr. Four subsequent additions of sodium hydroxide (500 ml. of 30% w/v solution) and dimethyl sulphate (250 ml.) were made over 6-hr. periods under nitrogen at room temperature with vigorous stirring, and then the product was isolated by dialysis against running tap water (2 weeks) and freeze-drying (11·5 g., Found: OMe, 19·0. Calc. for a sulphate-free product; OMe, 34·2). This product (10·8 g.) was dissolved in water and remethylated in the same way as before (13 further additions of reagents). The freeze-dried material (10·5 g.) was extracted with chloroform, and the extracts poured into light petroleum (b. p. 40—60°; 7 vol.). The precipitate was isolated on the centrifuge and dried *in vacuo* at 40° (6·2 g.; 59%, calculated on the the basis of a percentage purity for porphyran that was worked out from the analytical figures; Found: OMe, 31·0; $SO_3$, 1·5%). Further methylation with silver oxide in dimethylformamide [18] and in *N*-methyl-2-pyrrolidone [19] did not increase the methoxyl content of the chloroform-soluble fraction. An infrared spectrum (Nujol mull) showed a trace of hydroxyl absorption at 3400—3600 $cm.^{-1}$. A quantity (3·8 g.) of insoluble material remained after the chloroform extraction; the investigation of this is described below.

*Examination of the Products of Complete Fragmentation of Methylated Alkali-modified Porphyran.*—The polysaccharide was hydrolysed with 45% formic acid at 100° for 16 hr. After removal of the formic acid *in vacuo*, the residue was dissolved in water and examined by paper chromatography using solvent (B). By far the major products were 2,4,6-tri-*O*-methylgalactose and degradation products of 3,6-anhydrogalactose derivatives. Spots corresponding to 2,6- and 2,4-di-*O*-methylgalactoses were detected in small amounts, in roughly equal concentrations. Barely detectable amounts of 2,3,6-tri-*O*-methylgalactose were present.

In a separate experiment, the polysaccharide (10 mg.) was heated at 67° in a sealed tube (35 hr.) with methanolic hydrogen chloride (3%). After neutralisation (silver carbonate), filtration and evaporation, the mixture of products was examined by g.l.c. on the butanediol succinate polyester column. Derivatives of 2,4,6-tri-*O*-methylgalactose and 3,6-anhydro 2-*O*-methylgalactose were detected, but no derivatives of 3,6-anhydro-2,4-di-*O*-methylgalactose or of non-methylated 3,6-anhydrogalactose. The only difference from the gas chromatogram of a methanolysate of the glycoside (VIII) was the presence of a very small peak probably due to traces of 2,3,6-tri-*O*-methylgalactosides.

*Methanolysis of Methylated Alkali-modified Porphyran and Column Separation of the Products.*—The methylated polysaccharide (1·04 g.) was subjected to partial methanolysis with 3% methanolic hydrogen chloride (150 ml.) which had been prepared from methanol freshly distilled from magnesium methoxide. After 6 hr. at 37° under rigorously anhydrous conditions, the residue was removed on the centrifuge and re-methanolysed in the same way. After a third methanolysis virtually all the polysaccharide had dissolved (weight of residue, 0·007 g.). The solutions were combined, neutralised with silver carbonate, filtered, and concentrated to dryness. The residual syrup was treated again with dry methanolic hydrogen chloride (3%; 3·5 hr. at 37°), and after neutralisation ($Ag_2CO_3$) the solution was concentrated again to a syrup. A small portion (1 mg.) was acetylated for examination by gas chromatography; the major product was the methyl β-glycoside of tetramethylagarobiose (VIII) with only a small amount of the dimethylacetal (VI).

The mixture of products was separated by chromatography on a charcoal column. The column (30 cm. × 3·9 cm. diam.) was prepared using May and Baker's "Charcoal Decolourising" and Celite (3 : 2 by weight). After application of the syrup dissolved in the minimum volume of water, the column was eluted with aqueous butan-2-one of increasing concentration as indicated below. Fractions (50 ml.) were collected automatically and a sample (2 ml.) of every third one was analysed with the phenol–sulphuric acid reagents.[20] On the basis of these

[18] R. Kuhn, H. Trischmann, and I. Low, *Angew. Chem.*, 1955, **67**, 32.
[19] B.P. 806,935 (*Chem. Abs.*, 1959, **53**, 21,697).
[20] M. Dubois, K. A. Gilles, J. K. Hamilton, P. A. Rebers, and F. Smith, *Analyt. Chem.*, 1956, **28** 350.

results and of the analysis of selected fractions by g.l.c., the solutions were combined into larger fractions which were evaporated to dryness *in vacuo*. Each residue was dried further in a vacuum oven (30°) over phosphorus pentoxide and the methylated sugar extracted from most of the column material with which it was contaminated, with methanol. After filtration and evaporation to dryness, the fractions were found still to be contaminated to a small extent. Each one was therefore re-extracted with ethyl acetate; filtration and evaporation then gave the following products.

*Fraction A* (0·060 g.). This was eluted with 1% butane-2-one (0·75 l.). A strong Seliwanoff test was given in the cold, indicating the presence of breakdown products of 3,6-anhydrogalactose. Direct paper chromatography showed that a major product was a tri-*O*-methylgalactose. After hydrolysis (45% aqueous formic acid at 100° for 16 hr.) this same sugar was present together with products probably related to hydroxymethylfurfural, and small amounts of di-*O*-methylsugars. Thin-layer chromatography showed the absence of 3,6-anhydro-2-*O*-methylgalactose derivatives, and paper chromatography of the reduced hydrolysed fraction showed the absence of 3,6-anhydrogalactitol and its 2-*O* methyl ether. Fraction A probably therefore contains similar amounts of a reducing tri-*O*-methylgalactose and degradation products of 3,6-anhydro-2-*O*-methylgalactose, and represents the products of eliminative degradation of the reducing disaccharide (V) on the charcoal column.

*Fraction B* (0·025 g.). This was eluted with 1—1·5% aqueous butan-2-one (0·15 l.). G.l.c. showed a single major component, $T^{15}$ 7·5. This fraction gave a strongly positive Seliwanoff test indicating (within the context of the present investigation) the presence of a derivative of 3,6-anhydrogalactose. After hydrolysis with 0·01M-oxalic acid on a boiling-water bath for 1·5 hr., followed by neutralisation ($CaCO_3$), centrifugation, and reduction, the product was indistinguishable from 3,6-anhydro-2-*O*-methylgalactitol (recently synthesised in this laboratory [21]) on paper chromatography, and quite different from 3,6-anhydro-4-*O*-methylgalactitol (also recently synthesised [21]) and from 3,6-anhydrogalactitol. These tests indicate that the major component of this fraction is a methyl glycoside or dimethylacetal of 3,6-anhydro-2-*O*-methylgalactose.

*Fraction C* (0·055 g.). This was eluted with 1·5—3·0% aqueous butan-2-one (2·5 l.). G.l.c. on the butan-1,4-diol succinate column showed peaks having $T^{15}$ 4·07 and 4·62, corresponding closely to the values [15] for methyl 2,4,6-tri-*O*-methylgalactosides. Hydrolysis (45% formic acid at 100° for 6 hr.) followed by paper chromatography showed a major spot corresponding to 2,4,6-tri-*O*-methylgalactose, and quite distinct from 2,3,6-tri-*O*-methylgalactose, with a very minor spot corresponding to 2,3,4-6-tetra-*O*-methylgalactose. This fraction crystallised as needles, m. p. 58—68°, a range similar to that quoted [10] for the mixed methyl glycosides of 2,4,6-tri-*O*-methylgalactose. On the basis of this evidence, it is concluded that Fraction B is largely, if not quite entirely made up of methyl glycosides of 2,4,6-tri-*O*-methylgalactose.

*Fraction D* (0·047 g.). This was eluted with 1·5—3·0% aqueous butan-2-one (9·0 l.), after Fraction C. G.l.c. on the butan-1,4-diol succinate column showed the presence of 2,4,6-tri-*O*-methylgalactosides, but thin-layer chromatography suggested that degradation products from methanolysis were present in addition. Hydrolysis and paper chromatography showed 2,4,6-tri-*O*-methylgaclatose and some di-*O*-methyl and mono-*O*-methyl sugars, and also degradation products of 3,6-anhydrogalactose derivatives. It is concluded that Fraction D is a mixture of carbohydrate degradation products, 2,4,6-tri-*O*-methylgalactosides, and di- and tri-*O*-methyl disaccharide derivatives arising from a small degree of undermethylation of the polysaccharides and/or from the units carrying the small amount of extra sulphate not removed by alkaline elimination (see Discussion).

*Fraction E* (0·146 g.). This was eluted with 3—6% aqueous butan-2-one (8·0 l.). This fraction reduced Fehling's solution in the cold, and after methanolysis (3% methanolic hydrogen chloride at 100°, followed by neutralisation with silver carbonate and filtration) gave derivatives of 2,4,6-tri-*O*-methylgalactose and 3,6-anhydro-2-*O*-methylgalactose, as indicated by g.l.c. on the butan-1,4-diol succinate column (see above for *T* values). After reduction with excess of potassium borohydride in aqueous solution (48 hr.) and hydrolysis (45% formic acid at 100° for 16 hr.) paper chromatography showed the presence of 2,4,6-tri-*O*-methylgalactose and 3,6-anhydro-2-*O*-methylgalactitol only. On the basis of the above evidence, Fraction D appeared to be tetramethylagarobiose (V), and this was confirmed by conversion into the crystalline methyl β-glycoside (VIII) and also into the crystalline glycitol (VII). To prepare

[21] D. B. Alexander, T. C. S. Dolan and D. A. Rees, unpublished results.

the β-glycoside, a portion of Fraction D (0·066 g.) was treated with 3% methanolic hydrogen chloride for 30 min. at 50°, and then the solution was neutralised ($Ag_2CO_3$), centrifuged, and evaporated to dryness. Drying in a vacuum oven at 50° for 24 hr. appeared (thin-layer chromatography) to increase the proportion of β-glycoside in the mixture. The desired product was separated by preparative thin-layer chromatography on plates (20 × 20 cm.) coated with silica gel G, using a double development with butan-2-one–ether (1 : 2). The bands were detected by brief exposure to iodine vapour, and the band containing the β-glycoside was scraped from the plate and extracted with ethyl acetate. The product (0·011 g.), after recrystallisation from ethyl acetate had m. p. and mixed m. p. 147—149: (Kofler hot-stage). For conversion into *tetra-O-methylagarobi-itol* (VII), a portion of Fraction D (0·05 g.) was reduced with excess of potassium borohydride in aqueous solution (48 hr.). Excess of borohydride was decomposed by the addition of dilute acetic acid, and the solution was treated with Amberlite IR-120 resin ($H^+$ form) to remove potassium ions. The solution was evaporated to dryness, and methanol distilled from the residue 4 times to remove boric acid. The product, which crystallised entirely, gave a single peak on gas chromatography after acetylation. After recrystallisation (twice) from ethyl acetate it had m. p. and mixed m. p. (with material prepared similarly from authentic methyl tetramethyl-β-agarobioside) 148—149° (Kofler hot-stage), $[\alpha]_D^{17}$ −42° (*c* 1·1 in MeOH) (Found: C, 49·6; H, 7·7; OMe, 34·1%; *M*, 363. $C_{16}H_{30}O_{10}$ requires C, 50·3; H, 7·9; OMe, 32·5%; *M*, 382).

*Fraction F* (0·053 g.). This was eluted with 6—7% aqueous butan-2-one (1·4 l.). This was a small fraction, and was therefore only examined by g.l.c. after acetylation, which indicated that it contained the dimethylacetal (VI), β-glycoside (VIII), and the free sugar (V) in similar amounts.

*Fraction G* (0·493 g.). This was eluted with 7—14% aqueous butan-2-one (11·3 l.). This crystallised entirely on evaporation to dryness, and after recrystallisation (twice) from ethyl acetate had m. p. and mixed m. p. [with authentic glycoside (VIII) kindly provided by Professor Araki [10]] 140—142° (uncorrected value with capillary apparatus), $[\alpha]_D$ +39·3° (*c* 1·2 in $H_2O$). The *X*-ray powder diagram was identicalwith that of authentic material.

*Fraction H* (0·116 g.). This was eluted with water saturated with butan-2-one (4·5 l.). Acetylation and g.l.c. showed that simple agarobiose derivatives were absent. However, methanolysis (3% methanolic hydrogen chloride at 50° for 30 min.) followed by hydrolysis (0·01M-oxalic acid at 100° for 2·5 hr.), neutralisation ($CaCO_3$), reduction (excess of $KBH_4$ for 24 hr.), filtration, and removal of salts by treatment with IR-120 resin, evaporation to dryness, and distillation of methanol from the residue, gave a partly crystalline syrup. Acetylation and gas chromatography showed a single peak corresponding to the acetate of tetra-*O*-methylagarobi-itol. Hydrolysis and paper chromatography showed 2,4,6- and 2,3,6-tri-*O*-methylgalactoses in the approximate ratio 9 : 1, as well as degradation products of 3,6-anhydrogalactose derivatives and small amounts of tetra- and di-*O*-methyl galactoses. Fraction H would therefore seem to consist of high-molecular-weight oligosaccharides containing tetra-*O*-methylagarobiose units; a small proportion of these probably contain some 2,3,6-tri-*O*-methyl-L-galactose in place of 3,6-anhydro-2-*O*-methyl-L-galactose.

*The Repeating-unit Content of Methylated Alkali-modified Porphyran.*—Duplicate samples (about 30 mg.) were weighted into small flasks containing 3% methanolic hydrogen chloride (15 ml.). After heating at 37° for 6 hr., the solutions were centrifuged, and the residue was treated again in the same way a further four times. The final residue was weighed (3—5 mg.). The combined solutions were neutralised ($Ag_2CO_3$), centrifuged, and concentrated to dryness. The syrupy residues were dried briefly *in vacuo* and re-methanolysed with more methanolic hydrogen chloride (25 ml. for 9 hr.). After neutralisation, filtration, and evaporation, the concentration of disaccharide derivatives was determined by conversion into the corresponding mixture of glycitols. This was achieved by heating the residue with aqueous oxalic acid (0·01M; 10 ml.) at 100° for exactly 2 hr., followed by neutralisation ($CaCO_3$), reduction (excess of $KBH_4$ for 20 hr.), filtration, and evaporation. The product was mixed with internal standard, acetylated, and chromatographed as described above. Reference to a linear calibration graph, prepared using mixtures of crystalline tetra-*O*-methylagarobi-itol and standard, showed that the yield of tetra-*O*-methylagarobi-itol from the polysaccharide was 61%. The β-glycoside (VIII), after methanolysis for 15 hr., gave 74% of the glycitol; and a sample of β-glycoside which was treated very briefly with methanolic hydrogen chloride (3% at 37° for 10 min. until the optical rotation was constant) to effect conversion into the equilibrium mixture of glycosides

and dimethylacetal with the minimum of other changes, gave 81% of glycitol. These data were used to calculate the figures mentioned in the Discussion.

*Examination of the Insoluble Material Remaining after Purification of Methylated Alkali-modified Porphyran by Chloroform Extraction.*—This fraction contained 43% ash and 13·8% OMe. Hydrolysis and paper chromatography, and complete methanolysis and g.l.c., showed essentially the same mixture of products as was obtained by hydrolysis of the chloroform-soluble material (see above), together with small amounts of other products that may have been methylated xyloses from a contaminating polysaccharide. Partial methanolysis (3% methanolic HCl at 37° for 3 × 15 hr.), followed by acetylation and examination of the products by g.l.c., showed the pattern of peaks to be expected from a mixture of tetra-*O*-methylagarobiose derivatives. It would appear that this fraction contains methylated alkali-modified porphyran in association with inorganic material.

*Proton Magnetic Resonance Spectra.*—These were recorded using a Perkin-Elmer R10 (60 Mc./sec.) nuclear magnetic resonance spectrometer with tetramethylsilane as an internal standard. No useful information could be obtained from the spectrum of the glycoside (VIII) in deuterochloroform, nor from that of the glycitol (VII) in deuterium oxide. In the latter case the DOH signal partially obscured the signal from the anomeric proton. However, a solution of the glycitol in deuterochloroform gave a satisfactory spectrum in that a low-field doublet (5·57 τ) corresponding (integrated spectrum) to one proton could be assigned to the anomeric proton. Further comment is made on this spectrum in the Discussion.

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