The Structure and Biosynthesis of Porphyran: a Comparison of some Samples

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Porphyran (Peat & Rees, 1961), the main polysaccharide of the marine red alga *Porphyra umbilicalis* (L.) Kütz f. *laciniata* Lightf. has been shown to contain residues of D-galactose, L-galactose, 3,6-anhydro-L-galactose, 6-*O*-methyl-D-galactose and ester sulphate (Peat, Turvey & Rees, 1961), some of the ester being present as 1→4-linked L-galactose 6-sulphate (Turvey & Rees, 1961; Turvey & Williams, 1961; Rees, 1961*a*). These components do not occur in simple whole-number proportions relative to one another, and this at first suggests either that porphyran is a mixture of polysaccharides or that its structure is not a simple repeating unit. The failure of fractionation experiments to resolve porphyran suggests that no gross heterogeneity exists (Peat *et al.* 1961), but the possibility remains that it is a mixture of related polysaccharides.

The present paper describes a fresh approach to the problem of defining porphyran as a chemical entity. Samples of the polysaccharides from different *Porphyra* species, showing seasonal and environmental variations, have been examined in an attempt to assess the degree of variation of the composition of the polysaccharides. It is hoped that this will lead towards assigning a structure to porphyran and a biosynthetic scheme for it. Some progress has recently been made by other methods (Turvey & Williams, 1961), and this is discussed below.

EXPERIMENTAL

Polysaccharide extraction. The air-dried algal specimen (up to 20 g.) was extracted three times for 2 hr. periods with 1 l. portions of water, the mixture (which remained at pH 6–7) being kept simmering over a Bunsen burner. The extracts were obtained by straining through muslin and combined, freed from solid matter by decantation, concentrated to 20–50 ml. in a rotary film evaporator under diminished pressure at 35°, dialysed exhaustively and freeze-dried to a white or slightly-buff amorphous material. Sample 5 in Table 3 was further purified through the cetylpyridinium salt (Peat *et al.* 1961).

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Analytical methods

All analyses were done in duplicate. All volumes of solutions of less than 1 ml. were transferred with self-zeroing blow-out glass micropipettes obtained from H. J. Elliot Ltd., Treforest, Glamorgan. After further drying *in vacuo* over phosphorus pentoxide at 60° for 16 hr., a portion of the freeze-dried product from each algal specimen (50–100 mg.) was weighed out and dissolved in water (10·0 ml.). Samples of this solution were taken for the following determinations.

3,6-Anhydrogalactose content. This was estimated by treating a portion (25 μl.) with the resorcinol reagent of Yaphe (1960), the extinction of the resulting solution being measured in an EEL photoelectric colorimeter, with filter slide no. 623 (maximum transmission at 495 mμ).

1→4-Linked L-galactose 6-sulphate content (labile-sulphate content). This was estimated by treating a portion of the solution (2 ml.) with potassium tetrahydroborate (borohydride) and sodium hydroxide (Rees, 1961*a*), the amount of labile sulphate being calculated from the 3,6-anhydrogalactose formed.

Total sulphate content. A portion of the solution (100 μl.) was evaporated to dryness in a hard-glass test tube (⅝ in. × 6 in.) in the presence of sodium chloride (1 mg.) in an oven at 100–110°. Concentrated sulphate-free nitric acid (5 drops) was added to the residue, and the tube sealed and replaced in the oven for at least 12 hr., after which time it was cooled and opened. The tube was again placed in the oven and left until the contents had no detectable odour of nitric acid. The residue was taken up in water (1·00 ml.) and the sulphate present determined by the spectrophotometric method of Jones & Letham (1954). This procedure is now preferred to the alternative gravimetric method (Peat *et al.* 1961) both because it is quicker and because the latter method is unreliable in the presence of the silicious matter which often contaminates porphyran and which is difficult to remove (J. R. Turvey & D. A. Rees, unpublished observations; cf. Frei & Preston, 1961). Although the accuracy of the gravimetric method can be improved by filtering the solution before precipitation of the barium sulphate, the procedure is then even more time-consuming.

Galactose and 6-O-methylgalactose contents. These were estimated simultaneously as follows: A portion of the polysaccharide solution (250 μl.) was transferred to a hard-glass test tube (⅝ in. × 3 in.) containing hydrochloric acid (3·0N; 25 μl.) and the tube sealed and heated in a boiling-water bath for 12–16 hr. After being cooled, the tube was opened and part of the contents (25 μl.) transferred to a chromatogram (Whatman no. 1 paper) in small portions of about 3 μl. at a time. After each application the paper was

dried in a jet of cold air, since warm air causes some decomposition of the sugars in the acid solution. The use of cold air gave no such decomposition, since quantitative recoveries were obtained when standard mixtures of galactose and 6-*O*-methylgalactose in 0·5N-hydrochloric acid were analysed in this way. After the paper had been irrigated for 15 hr. with ethyl acetate–acetic acid–formic acid–water (18:3:1:4, by vol.), the sugars were estimated with aniline phthalate by the method of Wilson (1959).

RESULTS AND DISCUSSION

Tables 1–4 show that there is wide variability in the composition of the polysaccharide extracts: 3,6-anhydrogalactose may be present in amounts from 5 to 19%, ester sulphate from 6 to 11%, 6-*O*-methylgalactose from 3 to 28% and galactose from 24 to 45%. Certain regularities, however, are apparent: first, although the sulphate content is variable the ester seems always to be predominantly of the labile type, i.e. 1→4-linked L-galactose 6-sulphate; secondly, the molar percentages of sugars present are interrelated in a special way. This molar composition of each sample has been derived (*a*) by converting each percentage into a molar proportion through division by the appropriate molecular weight, (*b*) by expressing the molar proportions of the anhydride and the labile sulphate as percentages of the total molar proportions of sugars only. The molar composition is a more reliable guide to real variation in the polysaccharide than percentage composition, since the latter is influenced by the amount of inert material present (such as silicious substances) as well as by the nature of the cations associated with the sulphate groups.

With such molar percentages, although that of 3,6-anhydro-L-galactose varies between 12 and 29, and that of L-galactose 6-sulphate between 17 and 37, the sum of the two quantities varies only between 43 and 55 (and indeed all except three of the values lie between 45 and 53) with a mean of 48 (see Tables 1–4). The error in the sugar estimations is expected to be ±3–4%, and in calculating the molar percentages the uncertainty becomes ±6–8%, since these are quotients. The variation in 'total L-galactose' is thus almost within experimental error, suggesting that 3,6-anhydro-L-galactose and L-galactose 6-sulphate are interchangeable between the polysaccharides, and that an increase in the 3,6-anhydride content takes place at the expense of the 6-sulphate. Such a conclusion supports the hypothesis (cf. Rees, 1961*b*) that L-galactose 6-sulphate is the biological precursor of 3,6-anhydro-L-galactose. So far this hypothesis has rested on the characterization of an enzyme in *Porphyra* extracts which can convert the sulphate into the anhydride. Since it is currently accepted that caution is necessary in postulating

Table 1. *Variation in samples from species of* Porphyra *gathered at one season*

The following molecular weights are used in calculating the percentage composition values in this and the other Tables: for galactose, 162; for 6-*O*-methylgalactose, 176; for 3,6-anhydrogalactose, 144; for sulphate, 80.

Species	Month sampled	Place	Percentage composition					Molar ratios (×100)		Molar composition	
			3,6-Anhydrogalactose *A*	Labile sulphate *B*	Total sulphate	Galactose *C*	6-*O*-Methylgalactose *D*	*B*/*C*	*D*/*A*	*A*	*B*
P. linearis	Early March	Islay, Hebrides	5·1	8·3	9·3	41·8	3·1	40	50	12	33
P. linearis	Late March	Islay	5·6	8·7	9·2	40·7	4·7	43	74	12	34
P. umbilicalis (high-level forms)	April	Portincross, Ayrshire	12·2	9·1	10·3	36·8	20·3	50	138	20	27
P. umbilicalis (mid-shore form)	May	Portincross	10·7	10·6	10·2	41·8	15·5	51	119	18	31
P. umbilicalis f. *laciniata*	April	Portincross	14·2	7·5	8·3	27·2	22·6	56	133	25	24
P. leucosticta	May	Islay	18·8	9·7	9·7	45·1	10·6	43	47	28	25

Table 2. *Seasonal variation in* Porphyra umbilicalis *f.* umbilicalis

Sample	Date of collection	Nature of site	Percentage composition: 3,6-Anhydro-galactose *A*	Labile sulphate *B*	Total sulphate	Galactose *C*	6-*O*-Methyl-galactose *D*	Molar ratios (×100): *B*/*C*	*D*/*A*	Molar composition: *A*	*B*
High-level forms											
1	13. ii. 61	Exposed rocky reefs (Portincross, Ayrshire)	11·6	6·7	8·6	36·8	14·9	37	106	21	22
2	26. iii. 61		10·6	8·9	9·1	29·0	14·1	62	110	22	33
3	8. iv. 61		12·2	9·1	10·3	36·8	20·3	50	138	20	27
4	18. vi. 60	Exposed rocky headland (Farland Head, Millport)	15·9	8·8	9·1	34·7	23·8	51	123	24	24
5	12. viii. 60	Open rocky coast (Peggy's Cove, Halifax, N.S.)	13·2	8·0	8·1	37·6	16·6	43	105	22	24
6	4. x. 61 (*a*)	Exposed rocky reefs (Portincross)	12·6	7·6	8·1	33·1	20·4	47	133	22	23
7	4. x. 61 (*b*)		13·0	7·4	7·5	33·9	19·7	45	125	22	23
8	1. xi. 61 (*a*)		13·5	7·5	8·1	33·6	19·4	44	117	23	23
9	1. xi. 61 (*b*)		13·4	7·3	8·2	34·2	18·7	44	115	23	22
Mid-shore forms											
10	25. v. 61	Open coast, rocky reefs, wide zone of mussels (Portincross)	10·7	10·6	10·2	41·8	15·5	51	119	18	31
11	18. vii. 60	Open, boulder beach (Buckie, Aberdeen)	13·0	7·0	7·0	33·2	20·1	43	128	22	22
12	10. viii. 60	Harbour wall, open conditions (Halifax, N.S.)	10·4	9·6	9·5	34·3	13·5	56	106	20	33
13	4. x. 61	Open coast, rocky reefs, wide zone of mussels (Portincross)	12·6	7·6	8·1	33·1	20·4	47	133	22	23
14	4. x. 61	Boulders at mid-shore, sandy bay (Ardneil, Ayrshire)	9·0	6·8	6·7	27·4	17·6	50	158	19	26
15	1. xi. 61	Open coast, rocky reefs, wide zone of mussels (Portincross)	13·4	7·3	8·2	34·2	18·7	44	115	23	22

Table 3. *Seasonal variation in* Porphyra umbilicalis *f.* laciniata

Sample	Date collected	Nature of site	Percentage composition: 3,6-Anhydro-galactose *A*	Labile sulphate *B*	Total sulphate	Galactose *C*	6-*O*-Methyl-galactose *D*	Molar ratios (×100): *B*/*C*	*D*/*A*	Molar composition: *A*	*B*
1	8. ii. 61	Open, sandy bay (Kames, Millport)	12·5	5·0	6·8	24·2	17·8	42	117	26	19
2	8. iv. 61	Open, sandy bay (Ardneil, Ayrshire)	14·2	7·5	8·3	27·2	22·6	56	133	25	24
3	2. v. 61	Open, sandy bay (Kames)	11·7	8·7	10·1	34·0	18·0	52	126	21	28
4	18. v. 60		10·2	6·6	6·3	23·4	14·5	57	116	24	28
5	—. vi. 59	Boulders in open sandy bay (Aberdesach, N. Wales)	12·9	8·5	9·8	37·7	28·4	47	182	18	27*
6	6. ix. 60	Sheltered, boulder beach (Guernsey)	9·0	9·8	9·9	43·7	4·2	45	39	17	34
7	15. x. 60	Open, sandy bay (Ardneil)	12·1	8·8	7·9	33·4	17·3	53	117	22	28
8	1. xi. 61		17·5	5·6	7·6	26·6	24·6	42	115	29	17
9	26. xi. 60		11·8	9·1	8·7	33·4	19·6	55	135	21	28
10	26. xi. 60		13·6	8·4	8·6	33·2	17·7	57	107	24	26

* Corrected to include a small amount of unsulphated L-galactose (see Discussion).

Table 4. *Environmental variations in* Porphyra perforata (*Pacific coast*)

Date collected	Nature of site	Percentage composition: 3,6-Anhydro-galactose *A*	Labile sulphate *B*	Total sulphate	Galactose *C*	6-*O*-Methyl-galactose *D*	Molar ratios (×100): *B*/*C*	*D*/*A*	Molar composition: *A*	*B*
23. vii. 60	Mid-shore of sheltered harbour (Vancouver, B.C.)	7·0	8·5	9·3	36·5	10·9	47	127	15	31
30. vi. 60	Mid-shore in sheltered inlet (Monterey, Calif.)	7·5	8·2	7·9	37·5	10·9	44	123	15	30
12. vii. 60	Mid-shore, sheltered inland waters (Friday Harbor, Wash.)	7·6	9·8	9·8	39·9	7·1	50	76	15	37
23. vii. 60	Upper shores of sheltered harbour (Vancouver)	8·7	9·0	9·3	40·7	11·0	45	105	16	30
30. vi. 60	Mid-shore on open shore (Monterey)	10·3	10·3	9·8	40·9	15·8	51	124	18	31
30. vi. 60		10·8	9·5	9·6	44·0	14·1	44	108	18	28
30. vi. 60	Upper levels on open shore (Monterey)	11·0	10·4	9·1	38·3	16·4	53	122	19	31
30. vi. 60		11·3	8·7	8·1	35·4	16·2	50	117	20	28
10. vii. 60	Mid-levels of very exposed open coast (Vancouver Is., B.C.)	11·7	8·4	8·6	33·5	14·2	51	100	22	28
10. vii. 60	Upper levels of very exposed open coast, plants slightly protected from direct wave action (Vancouver Is.)	12·0	11·0	11·2	42·2	19·5	53	134	18	30

the nature of normal metabolic processes from the characterization of enzymes, this supporting evidence is welcome.

A relationship similar to that between 3,6-anhydrogalactose and its precursor must also exist between 6-*O*-methyl-D-galactose and D-galactose, because the sum of the molar percentages of these components almost always constitutes (by difference) 47–55 % of the polysaccharide, no matter how each amount varies individually. This confirms the conclusion reached by Turvey & Williams (1961), on the basis or partial-acid-hydrolysis studies, that the D-galactose and 6-*O*-methyl-D-galactose units in porphyran are structurally equivalent.

Turvey & Williams (1961) further suggest that the simplest view of the structure of porphyran which is consistent with their results is a chain of alternating (1→3)-β-linked D-galactose and (1→4)-α-linked L-galactose units, some of the D-galactose units being 6-*O*-methylated and the L-galactose occurring either as the 6-sulphate or the anhydride. The results of our survey are consistent with this structure, since there is now seen to be a widespread 1:1 correlation between the amounts present of L-galactose derivatives (these being apparently structurally interchangeable) and D-galactose derivatives (these also being apparently structurally interchangeable).

Although the analytical values in the Tables do not account for all of the sample weight taken, and therefore the presence of other polysaccharide components cannot be rigidly excluded, it is thought probable that the remainder is made up of (i) cations associated with the sulphate groups, (ii) silicious material, and (iii) moisture, which is very difficult to remove from sulphated polysaccharides even by prolonged drying *in vacuo* at elevated temperature. Sugars other than those listed in the tables have been found only in trace quantities on paper chromatograms of acid hydrolysates, and it therefore seems that the analyses presented here account for virtually all of the polymeric carbohydrate extracted by water from each algal specimen. The typical polysaccharide material in hot-water extracts from members of the genus *Porphyra* is thus made up of nearly equal amounts of L-galactose derivatives and of D-galactose derivatives, and we suggest that the term 'porphyran' should be taken to refer to this material. Since the polysaccharide differs in composition from sample to sample, and moreover since any one sample is not necessarily homogeneous, porphyran should be regarded as a family of closely related polysaccharides rather than as a structurally unique entity. Evidence for the heterogeneity of sample 5 in Table 3 has been obtained by Dr J. R. Turvey (personal communication) who has resolved it into two components in the preparative ultracentrifuge. The component which sedimented rapidly had 25 % of 3,6-anhydrogalactose and 4·1 % of esterified sulphate, whereas the other component had 11·5 % of 3,6-anhydrogalactose and 9·4 % of esterified sulphate.

The discussion above is based on the assumption that there is little L-galactose that occurs free, rather than as the 6-sulphate or 3,6-anhydride. This has been checked in only one instance (that of sample 5 in Table 3), when it was found that 9 % of the total L-galactose was in fact free. This sample was older and more extensively purified than the others, and it is not clear to what extent this free L-galactose arose from units that were previously sulphated, during working up and ageing. However, it is likely that small variable amounts of free L-galactose occur in all samples, and this might partially account for the observed spread in the values for 'total L-galactose'.

No constant ratio exists throughout Tables 1–4 between any two of the individual components of porphyran, and it therefore seems that, apart from maintaining equivalence between the amounts of D- and L-galactose derivatives present, the porphyran-synthesizing enzyme system does not assemble the units in a rigidly predetermined manner, i.e. the synthesis is to some extent flexible. The enzyme that is believed to be responsible for 3,6-anhydrogalactose synthesis (Rees, 1961*b*) can operate without any necessary changes other than sulphate release taking place. These results must be considered when a mechanism is proposed for incorporation of the 6-*O*-methylgalactose into the polymer. However, certain rather loose correlations exist (Tables 1–4). The amount of 6-*O*-methylgalactose present does not usually vary very much relative to 3,6-anhydrogalactose, the ratio normally being between 1:1 and 1:1·4; similarly, the ratio galactose:sulphate is usually in the range 1:0·4 to 1:0·6. There is gross variation in the ratio galactose:3,6-anhydride (1:0·15 to 1:0·6) and in the ratio sulphate:methylgalactose (1:0·3 to 1:5). The most pronounced variation in porphyran composition from sample to sample is usually therefore between the proportion of 3,6-anhydrogalactose and 6-*O*-methylgalactose on the one hand, and D-galactose and L-galactose 6-sulphate on the other.

Factors influencing porphyran composition. Preliminary analyses of a number of porphyran samples from different species of *Porphyra* collected from Scottish shores in the spring (Table 1) emphasized the variation between the samples. Seasonal samples were therefore examined of the forms *Porphyra umbilicalis* f. *umbilicalis* and *P. umbilicalis* f. *laciniata* (Tables 2 and 3). Again there is no marked pattern; sample 8 (Table 3) shows a remarkably high amount of 3,6-anhydro-

galactose, and this was extracted from very young plants; sample 6 (Table 3) and sample 14 (Table 2) show low values of 3,6-anhydrogalactose, and both of these came from deteriorating plants, sample 6 from plants that were much bleached by insolation and sample 14 from old and tattered plants after a period of stormy weather. Correlation with the environment is suggested by Table 4, which shows the analyses of porphyran samples from collections of *P. perforata* made about the same time from a number of sites on the Pacific coast of North America. The molar percentages of 3,6-anhydrogalactose suggest that the greater the exposure the higher is the 3,6-anhydride content. Eppley & Cyrus (1960) have already suggested that one of the functions of porphyran might be to act as a cushion against physical buffeting; and it has been noticed in the present survey, as well as by Rees (1961*a*), that porphyran samples of high 3,6-anhydrogalactose content give thicker solutions and greater tendencies to form gels. Thus the amount of 3,6-anhydride present may be important as an adaptive ecological character.

SUMMARY

1. The water-soluble polysaccharides have been isolated and examined from about 40 different specimens of *Porphyra* species showing seasonal and environmental variations.

2. There is a wide variability in the composition of the polysaccharide extracts: 3,6-anhydro-L-galactose may be present in amounts from 5 to 19%, ester sulphate from 6 to 11%, 6-*O*-methyl-D-galactose from 3 to 28% and galactose from 24 to 45%.

3. Although the sulphate content is variable the ester always seems to occur predominantly as 1→4-linked L-galactose 6-sulphate.

4. Regularities in the analytical results suggest that 3,6-anhydro-L-galactose and L-galactose 6-sulphate are interchangeable between the polysaccharides, and that D-galactose and 6-*O*-methyl-D-galactose are related in a similar manner.

5. In all the polysaccharides isolated, 3,6-anhydro-L-galactose and L-galactose 6-sulphate total approximately half of the sugar units, the other half being made up of D-galactose and 6-*O*-methyl-D-galactose.

6. The implications of these and other aspects of the results for the homogeneity, structure and biosynthesis of porphyran are discussed.

7. A preliminary attempt has been made to correlate polysaccharide composition with variation in the algal specimens examined.

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Condensed Tannins

14. FORMATION OF (−)-3′,4′,5′,7-TETRAHYDROXYFLAVANONE AND (+)-3′,4′,5′,7-TETRAHYDROXYFLAVAN-4-OL BY INTERCONVERSION FROM (+)-DIHYDROROBINETIN, AND SYNTHESIS OF THEIR RACEMATES*

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The isolation of the isomeric compounds (−)-robtin [(−)-3′,4′,5′,7-tetrahydroxyflavanone] and robtein (2′,3,4,4′,5-pentahydroxychalcone) from the heartwood of *Robinia pseudacacia* suggested to Roux & Paulus (1962) that they originate successively from (+)-dihydrorobinetin [(+)-3′,4′,5′,7-tetrahydroxyflavonon-3-ol] as part of a more general scheme of flavonoid biogenesis based on the

* Part 13: Roux & Paulus (1962).