1022. *Estimation of the Relative Amounts of Isomeric Sulphate Esters in Some Sulphated Polysaccharides.*

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It is shown that suitably linked L-galactose 6-sulphate units in porphyran can be converted quantitatively by alkaline treatment into 3,6-anhydrogalactose. This treatment has been used to estimate the proportion of this sulphate in the polysaccharide, the conclusion being confirmed by periodate oxidation and infrared studies. The present sample of porphyran contains 9·8% of sulphate (as SO_3) of which 86% is present as 1,2- or 1,4-linked L-galactose 6-sulphate. The mucilage of *Dilsea edulis* has been re-examined by these techniques, and an earlier structure is revised to accommodate the new findings. Certain other galactan sulphates have been briefly examined.

The eliminations of carbohydrate sulphates in alkaline solution were elucidated by Percival,[1] and the principles were subsequently applied quite widely [2,3] in attempts to deduce the positions of sulphate ester groups in polysaccharides from the stability of the esters to alkali. Unfortunately, precautions were not usually taken in such structural work to avoid the release of sulphate by general destruction of the polysaccharide under the strongly alkaline conditions (usually boiling N-sodium hydroxide), and the results give at most a qualitative answer. When a proportion of the ester is labile to alkali it is often uncertain whether this is due to degradation of the polysaccharide or to more specific S_N2 elimination with resultant anhydro-ring formation.[1] The results of such studies have therefore been conclusive only when the new sugar units could be identified,[3a] or when it could be shown that the ester was so placed that it could not be eliminated. Methods have now been devised whereby alkaline elimination can be exploited more fully for the structural analysis of some seaweed polysaccharides.

Porphyran, the galactan sulphate of the marine red alga *Porphyra umbilicalis*,[4] was used as a model because it contains L-galactose 6-sulphate units which are so linked that according to Percival's rules the ester should be labile to alkali, giving 3,6-anhydrogalactose.[5] In preliminary experiments, the 3,6-anhydrogalactose present in the reaction mixture was measured, as an indication of the extent of both sulphate elimination and polysaccharide degradation. The reaction proceeded at a convenient rate in N-sodium hydroxide at 80°, but was attended by rapid destruction of the polysaccharide (Table, *a*).

Concentration of 3,6-anhydrogalactose (% of initial value) on treatment of porphyran with alkali.

Time (min.)	40	70	130	170	200	260	300
3,6-Anhydrogalactose (% of initial value) from:							
(*a*) porphyran	148	152	126		109		
(*b*) reduced porphyran	172	186		186		177	
(*c*) reduced porphyran + borohydride		193	215		215		215

Destruction of the polysaccharide was substantially less when porphyran was reduced with potassium borohydride before the reaction (Table, *b*), suggesting that the principal cause of degradation was " peeling " from the reducing end.[6] The fact that slight destruction occurred even after reduction indicated a certain amount of depolymerisation, probably exposing new reducing groups at which " peeling " could occur. This secondary degradation was avoided by including potassium borohydride in the actual reaction mixture,

[1] Percival, *Quart. Rev.*, 1949, **3**, 369.
[2] Buchanan, Percival, and Percival, *J.*, 1943, 51; Conchie and Percival, *J.*, 1950, 827; Fisher and Percival, *J.*, 1957, 2666.
[3] Barry and Dillon, *Proc. Roy. Irish Acad.*, 1945, **50**, *B*, 349.
[3a] Ricketts, *J.*, 1956, 3752; Overend and Ricketts, *Chem. and Ind.*, 1957, 632.
[4] Peat, Turvey, and Rees, *J.*, 1961, 1590; Turvey and Rees, *Nature*, 1961, **189**, 831.
[5] Rees, *Biochem. J.*, 1961, **81**, 347.
[6] Whistler and BeMiller, *Adv. Carbohydrate Chem.*, 1958, **13**, 289.

the new reducing groups presumably being protected under these conditions by immediate reduction (Table, *c*). The properties of the product isolated from the reaction confirmed that degradation had been minimised. It was non-dialysable, gave more viscous solutions than the original porphyran, and exhibited a tendency to gel out of solution (especially in the presence of salts) which was not shown by the original porphyran. The sulphate content was diminished (1·9%: originally 9·8%) and the anhydrogalactose content increased (30·0%; originally 12·9%). The product therefore resembles agar and κ-carrageenin in its high 3,6-anhydrogalactose content and in its tendency to form gels. Also there was molar correspondence between the sulphate released and the anhydro-sugar formed (104% recovery of 3,6-anhydrogalactose), confirming that all the sulphate release involved 3,6-anhydride formation.

These results cannot be used with confidence to calculate the proportion of 6-sulphate in porphyran, because 3,6-anhydrogalactose could also be given by galactose 3-sulphate units with a free position 6. However, only those 3,6-anhydrogalactose-producing units in which the sulphate is at position 6 will be attacked by periodate. It was found that porphyran reduced, on average, 0·40 mole of periodate for every hexose unit, and that when the oxidised polysaccharide was warmed with alkali under the usual conditions no increase in the 3,6-anhydrogalactose content was observed. It therefore seems that all the sugar sulphate residues giving rise to the 3,6-anhydride are in fact oxidised by periodate, *i.e.*, are 1,2- or 1,4-linked L-galactose 6-sulphate units, the L-configuration following because it has been established [4] that all the 6-sulphate, if not all the sulphate, is attached to L-galactose units.

The infrared spectrum of porphyran showed absorption bands thought to be characteristic of sulphate esters.[7] These were at 1240 (given by all sulphate esters), 820 (given by sulphated primary hydroxyl groups), and 850 cm.$^{-1}$ (given by sulphated secondary hydroxyl groups which are axial with respect to the sugar ring to which they are attached). The absorption at 820 cm.$^{-1}$ was strong, as would be expected since this represents (from the above evidence) at least 86% of the ester. This peak was absent from the alkali-treated porphyran, confirming once more that 6-sulphate was removed in 3,6-anhydrogalactose formation. The 850 cm.$^{-1}$ absorption was weak and was present in porphyran both before and after alkaline treatment, consistently with its having arisen from galactose 4-sulphate units which are stable to alkali in whatever glycosidic linkage.

These experiments show that 86% of the ester sulphate in this sample of porphyran is present as 1,2- or 1,4-linked L-galactose 6-sulphate, the 1,4-linkage being, for reasons already stated,[5] the more likely. The remainder of the sulphate might be attached to position 4 of galactose units, though further evidence for this is desirable. The content of L-galactose 6-sulphate is sufficient to account for 70% of the periodate uptake, so that most of the other units are apparently joined by 1,3-linkages. Since it has been shown that L-galactose 6-sulphate is probably the biological precursor of the 3,6-anhydrogalactose in native porphyran,[5] these two units are likely to be structurally equivalent, and taken together they represent one-third to one-half of the sugar units in the polysaccharide. A distinct resemblance therefore emerges between porphyran and certain other polysaccharides of the red algae, which contain approximately equal amounts of 1,3-linked D-galactose and 1,4-linked 3,6-anhydrogalactose, sometimes with position 4 of the D-galactose units sulphated.[8]

The Mucilage of Dilsea edulis.—Although a tentative structure has been put forward for this polysaccharide,[9] the previous authors emphasised that more work is necessary before a unique structure is possible. The evidence quoted concerning the location of

[7] Orr, *Biochim. Biophys. Acta*, 1954, **14**, 173; Lloyd, Dodgson, Price, and Rose, *ibid.*, 1961, **46**, 108; Lloyd and Dodgson, *ibid.*, p. 116.

[8] (*a*) O'Neill, *J. Amer. Chem. Soc.*, 1955, **77**, 6324; Araki, *Bull. Chem. Soc. Japan*, 1956, **29**, 543; (*b*) Yaphe, *Canad. J. Bot.*, 1959, **37**, 751.

[9] Barry and McCormick, *J.*, 1957, 2777.

the sulphate groups has been that (i) much of the ester is stable to alkali since " 50% of the combined sulphate is retained after 13 hours' boiling," [3] (ii) an unidentified relatively acid-stable substance, which gives 3,6-anhydrogalactosazone with phenylhydrazine, is present in the hydrolysate of the polysaccharide.[9] Barry and McCormick [9] pointed out that these two phenomena could be explained if the polysaccharide contained 1,3-linked galactose 6-sulphate units, since according to Percival's rules such a unit would be stable to alkali and some of the free sugar 6-sulphate would survive the conditions used for acidic hydrolysis to give 3,6-anhydrogalactosazone on subsequent treatment with phenylhydrazine. When the mucilage of *Dilsea edulis* was re-examined the following points emerged: (i) There is pronounced infrared absorption at 850 cm.$^{-1}$, with none detectable at 820 cm.$^{-1}$; it therefore appears that most of the sulphate is on galactose units at position 4. (ii) Warming the material with alkali increases the amount of 3,6-anhydrogalactose in the solution by an amount corresponding to 6% of the total sulphate, similar treatment of the oxidised polysaccharide leading to no such increase. A modification of Barry and McCormick's structure such as the following is therefore necessary. Two structurally dissimilar regions exist,[9] and these may be in the same or different molecules. The major component is a chain of 1,3-linked galactopyranose units (with the possibility of some branching), sulphate occurring on positions 4 of some of these. This structure takes account of the results of the Barry degradation studies by the earlier workers and explains the infrared results and the alkali-stability of most of the sulphate; this structure is similar to that of the λ-carrageenin studied by Morgan and O'Neill.[10] The second region is an alternating chain of 1,3- and 1,4-linked galactose units, some of the 1,3-linked units perhaps carrying sulphate at position 4 and some of these linked 1,4 occurring as the 6-sulphate or 3,6-anhydride. Xylose was originally supposed to occur in this region, but it now appears that this was part of a contaminating polysaccharide, since it is only present in traces in the present sample (purified by precipitation with Cetavlon). In the absence of further evidence the uronic acid is not placed in either region. This second structure is also a revision of that formerly put forward on the basis of Barry degradation studies; it accounts for the results of the present alkali and periodate studies and for the indications of 6-sulphate found by Barry and McCormick. It resembles that of certain other red seaweed polysaccharides (see the discussion of porphyran above).

Other Polysaccharides.—λ-Carrageenin has also been studied in this way: the preliminary results have been published.[11] Certain other seaweed polysaccharides, when warmed with alkali, showed small increases in 3,6-anhydrogalactose content, the extent corresponding in each case to the release of 2—5% of the total sulphate. Structural significance cannot be given to these observations until the homogeneity of the samples has been more closely examined, but together with the results already reported they show that units giving 3,6-anhydrogalactose on warming with alkali are common in the galactan sulphates of red algae. The specimens examined were: κ-carrageenin,[8] the water-soluble polysaccharides of *Eucheuma spinosum*, and the polysaccharides precipitated by potassium ions from extracts of *Furcellaria fastigiata* [12] and *Hypnea musciformis*.[8b] In all cases the infrared spectrum suggested, by the existence of a band at 850 cm.$^{-1}$, that the major sulphate ester was galactose 4-sulphate.

Experimental

The sample of porphyran, except where otherwise stated, was prepared by Mr. T. P. Williams, working in collaboration with Professor Stanley Peat, F.R.S., and Dr. J. R. Turvey, by extraction of seaweed harvested in June. It had 9·8% of sulphate (as SO_3) and 12·9% of 3,6-anhydrogalactose (anhydro-basis).

Analytical Methods.—Infrared spectra of polysaccharides were recorded with a Perkin–Elmer Infracord spectrophotometer and Nujol mulls. 3,6-Anhydrogalactose was determined

[10] Morgan and O'Neill, *Canad. J. Chem.*, 1959, **37**, 1201.

[11] Rees, *Chem. and Ind.*, 1961, 793.

[12] Painter, *Canad. J. Chem.*, 1960, **38**, 112; Clancy, Walsh, Dillon, and O'Colla, *Proc. Roy. Dublin Soc.*, 1960, **1**, *A*, 197.

by Yaphe's resorcinol method.[13] Determinations of total ester sulphate were carried out by destruction of the sample (about 1 mg.) with concentrated nitric acid at 100° in a sealed tube overnight, in the presence of sodium chloride (1 mg.). The nitric acid was boiled off and the last traces were removed by co-evaporation with concentrated hydrochloric acid, the residue was dissolved in water (1 ml.), and the sulphate determined spectrophotometrically with 4-amino-4′-chlorodiphenyl.[14]

General Procedure for Alkaline Treatment of Polysaccharides and Oxidised Polysaccharides.—The material, in 0·2—1·0% aqueous solution, was reduced with an excess of potassium borohydride at room temperature for 24 hr., and 3N-sodium hydroxide (0·5 vol.) was added together with a further quantity of potassium borohydride (about twice the weight of polysaccharide). A sample was withdrawn into a Pyrex test-tube which was sealed and heated on a water-bath at 80° for 2—4 hr. The tube was opened (care is necessary, because the tube contains hydrogen under pressure, which tends to ignite if the tube is opened by applying the molten tip of a glass rod to a scratch), and the 3,6-anhydrogalactose present in the contents compared with that in the remainder of the solution which had been kept at room temperature. In control experiments no change in 3,6-anhydrogalactose content was detected when such solutions were kept at room temperature for 24 hr. In examination of periodate-oxidised polysaccharides the reagents were re-calibrated with standards containing the same amount of iodide as the unknown, since iodide interferes slightly with the method.

Large-scale Alkaline Treatment of Porphyran.—Porphyran (1·61 g.) in water (250 ml.) was reduced at room temperature for 48 hr. with potassium borohydride (0·2 g.). Sodium hydroxide (10 g.) and potassium borohydride (3 g.) were dissolved in the solution, and the flask loosely stoppered and placed on a water bath at 80° for 2 hr. After this period the 3,6-anhydrogalactose content had risen to a value corresponding to 28·0% of the original material. The alkaline solution was neutralised (Amberlite IR-120 resin, H^+ form) and a portion placed in a dialysis bag in a beaker of stirred water. No 3,6-anhydrogalactose-containing material had passed through the membrane after 24 hr. (limit of detection, 1% of the amount present). The viscous solution was dialysed exhaustively against running tap water (3 days) and freeze-dried (1·24 g.; 1·87% of sulphate and 30·0% of 3,6-anhydrogalactose). From the analytical figures it is calculated that 1·61 millimoles of ester sulphate were eliminated in the reaction, with concomitant formation of 1·69 millimoles of 3,6-anhydrogalactose.

Periodate Oxidation of Porphyran.—Porphyran (16·9 mg.) was dissolved in 0·04M-sodium metaperiodate (10 ml.) and left in the dark at room temperature, the change in periodate concentration being followed spectrophotometrically.[15] Samples (1 ml.) were withdrawn into ethylene glycol (3 drops) for determination of residual galactose 6-sulphate by the method given above. The following results were obtained:

Time (hr.)	27	50	120
Periodate consumed (mole per hexose unit)	0·31	0·35	0·40
Galactose 6-sulphate remaining (%)	19	12	0

Examination of the Mucilage of Dilsea edulis.—This sample was kindly given by Drs. K. O. Lloyd and P. F. Lloyd. It was recovered from the residues from floridean starch preparation.[16] It had 0·9% of 3,6-anhydrogalactose and 15·0% of sulphate. Only traces of xylose were detected by paper chromatography of a complete acid hydrolysate. Treatment with alkali was carried out by the general method given above. The mucilage (12 mg.) was oxidised at room temperature in the dark in 0·05M-periodate (2·5 ml.). After 4 days, 94% of the units which originally gave 3,6-anhydrogalactose with alkali had been removed.

Other Polysaccharides.—The other polysaccharides mentioned in the discussion were kindly provided by Dr. W. Yaphe.[8b,13]

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[13] Yaphe, *Analyt. Chem.*, 1960, **32**, 1327.
[14] Jones and Letham, *Chem. and Ind.*, 1954, 662.
[15] Aspinall and Ferrier, *Chem. and Ind.*, 1957, 1216.
[16] Peat, Turvey, and Evans, *J.*, 1959, 3223.