# Carrageenans. Part VII.[1] Polysaccharides from *Eucheuma spinosum* and *Eucheuma cottonii*. The Covalent Structure of ι-Carrageenan

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ι-Carrageenan has been characterized by chemical methods to provide a firm basis for interpretation of data from *X*-ray diffraction and optical rotation, and so to confirm the carrageenan double helix. This polysaccharide was isolated from the red seaweed *Eucheuma spinosum* and shown by methylation analysis and partial fragmentation, and by other evidence, to have a masked repeating structure in which D-galactose 4-sulphate and 3,6-anhydro-D-galactose 2-sulphate residues are arranged alternately in linear chains, with formal replacement of approximately one in every ten anhydride residues by D-galactose 2,6-disulphate. Treatment with alkaline borohydride converts this structure to a genuinely alternating copolymer.

A polysaccharide from *E. cottonii* has similarly alternating 1,4- and 1,3-linked residues but is distinctive in that it has not detectable 2-sulphate. It therefore corresponds more closely than any other known natural polysaccharide to an idealized κ-carrageenan.

PHYSICAL measurements have recently revealed that certain carrageenans have spectacular conformational properties. *X*-Ray diffraction analysis of oriented specimens has shown[2] that κ- and ι-carrageenans can exist as double helices in the solid state. When these same polysaccharides are heated and cooled in aqueous solution they show optical rotation changes which are believed to represent the double-helix-to-coil transition.[3-5] The reversible association of κ-carrageenan with certain galactomannans can be regarded[6] as 'polysaccharide quaternary structure'. All this behaviour is new and unexpected for polysaccharides,[7] and suggests that polysaccharides might after all be valuable in advancing the general theory of polymer conformations. We now report the chemical characterization of two substances that could be useful for this purpose.

The polysaccharides were prepared from carefully hand-sorted *Eucheuma spinosum* and *Eucheuma cottonii* respectively, in the laboratories of Marine Colloids, Inc.[8] After hydrolysis of each with aqueous acid, galactose was the only sugar that could be detected by paper chromatography; however, travelling close behind the solvent front, were large amounts of sugar degradation products, suggesting the presence of 3,6-anhydrogalactose residues. Quantitative analysis showed that the relative proportions of galactose, its 3,6-anhydride, and ester sulphate, were 1·00 : 0·85 : 1·09 for the polysaccharide from *E. cottonii* and 1·00 : 0·85 : 1·94 for the polysaccharide from *E. spinosum*. Both gave additional 3,6-anhydride when warmed with alkaline borohydride. If this were formed in the usual elimination[9,10] from a stoicheiometric quantity of galactose sulphate it could be calculated that the compositions of the modified polysaccharides were 1·00 : 0·99 : 1·10 and 1·00 : 1·03 : 2·03 respectively; within experimental error these can be regarded as 1 : 1 : 1 and 1 : 1 : 2. Similar results were obtained when the sulphate elimination was performed with calcium hydroxide.[8] The i.r. spectra[8] can be interpreted by comparison with data for model glycosides and well characterized polysaccharides,[11] to show that galactose 4-sulphate residues are present in both, with additional sulphate at position 2 of each 3,6-anhydride

TABLE 1

Composition of *Eucheuma* polysaccharides calculated from the action of alkaline borohydride before and after periodate oxidation, and comparison with direct analysis

| | Percent of 4-linked residues that occurs as: galactose 6-sulphate | galactose 2,6-disulphate | Molar ratio galactose : 3,6-anhydride : sulphate[a] | Molar ratio from direct analysis |
|---|---|---|---|---|
| *E. cottonii* | 7·2 | 0 | 1·00 : 0·87 : 1·00 | 1·00 : 0·85 : 1·09 |
| *E. spinosum* | 0 | 9·5 | 1·00 : 0·82 : 1·99 | 1·00 : 0·85 : 1·94 |

[a] For this calculation, use is made of the knowledge from methylation analysis (this paper) that the 3,6-anhydride residues are fully sulphated in *E. spinosum* but nonsulphated in *E. cottonii*.

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[2] N. S. Anderson, J. W. Campbell, M. M. Harding, D. A. Rees, and J. W. B. Samuel, *J. Mol. Biol.*, 1969, **45**, 85.

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[7] D. A. Rees, 'The Shapes of Molecules: Carbohydrate Polymers,' Oliver and Boyd, Edinburgh, 1967, p. 113.

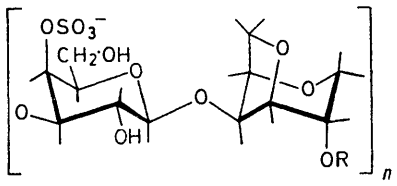
[8] Preliminary investigation, D. J. Stancioff and N. F. Stanley, in 'Proceedings of the 6th International Seaweed Symposium' ed. R. Margalef, Subsecretaria de la Marina Mercante—Direccion General de Pesca Maritima Madrid, 1969, p. 595.

[9] D. A. Rees, *J. Chem. Soc.*, 1961, 5168.

[10] D. A. Rees, *J. Chem. Soc.*, 1963, 1821.

[11] N. S. Anderson, T. C. S. Dolan, A. Penman, D. A. Rees, G. P. Mueller, D. J. Stancioff, and N. F. Stanley, *J. Chem. Soc.* (*C*), 1968, 602.

in the polysaccharide from *E. spinosum*. Experiments to be reported elsewhere [12] show that equimolar proportions of galactose and 3,6-anhydride are arranged alternately in the pattern that is now well known for many polysaccharides from red seaweeds.[13] The glycosidic linkages are therefore expected to be α-1,3 and β-1,4, and structures (I) and (II) may be proposed for the products of alkaline borohydride action on the

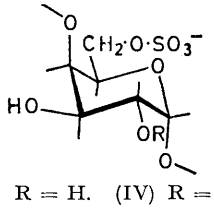



(I) R = H, κ-Carrageenan (idealized). (II) R = $SO_3^-$, ι-Carrageenan (idealized)

*E. cottonii* and *E. spinosum* polysaccharides, respectively. The measured optical rotations agree with these structures. Using the knowledge that sulphate ester usually makes little contribution to molecular rotation [14a] and that optical activity is related to the conformations about the bonds to each glycosidic oxygen atom,[14b] a value has been calculated for *E. spinosum* polysaccharide in the random coil form (+34°) which is in good agreement with experiment (+38°).[3] A similar calculation for *E. cottonii* polysaccharide now leads to a value (+45°) which is also in good agreement with experiment (+50°).

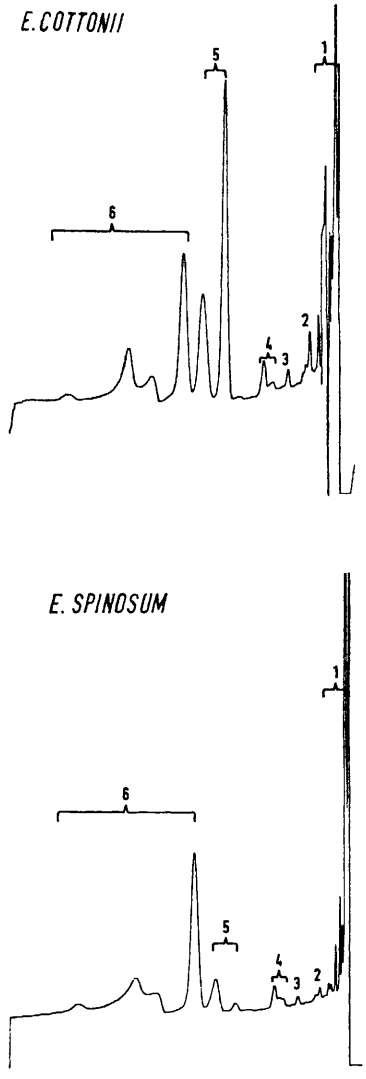
Structure (I) corresponds to κ-carrageenan,[15] and we have already proposed [2,4,13] that (II) be named ι-carrageenan.

Before treatment with alkaline borohydride, galactose sulphate evidently occurs in place of a proportion of 3,6-anhydride residues. For the polysaccharide from *E. cottonii*, these sulphate residues were found to be completely oxidizable by periodate, whereas those from *E. spinosum* were completely resistant. This indicates [9,10] the presence of galactose 6-sulphate (III) in the former and galactose 2,6-disulphate (IV) in the latter.




(III) R = H. (IV) R = $SO_3^-$

Gas chromatograms obtained after methylation and methanolysis of the two polysaccharides showed the expected similarities and differences (Figure). 2,6-Di-*O*-methylgalactosides were formed in large amounts from both, whereas the derivatives of 3,6-anhydro-2-*O*-methylgalactose were substantial products from *E. cottonii* polysaccharide only. The glycosides and dimethyl acetal of 3,6-anhydrogalactose itself were expected but not observed amongst the products from *E.*




Comparison of *Eucheuma* polysaccharides by methylation, followed by methanolysis and g.l.c. For conditions, see text. Peaks are assigned as follows: 1, solvent and degradation products; 2, 2,3,4,6-tetra-*O*-methylgalactosides; 3, 2,3,6-tri-*O*-methylgalactoside (the first of the three peaks expected from the glycosides of this sugar); 4, mixture of 2,3,6- and 2,4,6-tri-*O*-methylgalactosides; 5, 3,6-anhydro-2-*O*-methylgalactose derivatives; 6, 2,6-di-*O*-methylgalactosides

*spinosum* polysaccharide and were presumably lost by degradation under the conditions of methanolysis. Model experiments with methyl 3,6-anhydro-α-D-galactopyranoside and its 2-*O*-methyl ether confirmed that the two anhydride residues differ in their ease of destruction.

The identification of 2,6-di-*O*-methylgalactose was

[12] C. J. Lawson, D. A. Rees, D. J. Stancioff, and N. F. Stanley, following paper.
[13] D. A. Rees, *Adv. Carbohydrate Chem. Biochem.*, 1969, **24**, 267.
[14] (*a*) M. J. Harris and J. R. Turvey, *Carbohydrate Res.*, 1970, **15**, 51; (*b*) D. A. Rees, *J. Chem. Soc.* (*B*), 1970, 877.
[15] N. S. Anderson, T. C. S. Dolan, and D. A. Rees, *J. Chem. Soc.* (*C*), 1968, 596.

confirmed by hydrolysis and preparative chromatography, which gave this sugar in the crystalline state from both polysaccharides and allowed it to be characterized as the D-enantiomer. As minor products from this separation, 2,4,6- and 2,3,6-tri-*O*-methylgalactose were both isolated. Presumably these are derived from a minor proportion of nonsulphated galactose residues in each polysaccharide structure. The 4-linked residues of this type are particularly important because they are subject to Smith degradation to give polysaccharide segments with useful conformational properties.[4,6,16,17] We presume that 6-sulphate elimination occurred as a side reaction during methylation and that it was for this reason that 2,3-di-*O*-methylgalactose was not detected amongst the products from *E. cottonii*, as would have been expected otherwise. Some mono-*O*-methylgalactoses were detected from both polysaccharides and are presumed to include products of incomplete methylation.

TABLE 2

Yields of sugars from the methylation analysis of *Eucheuma* polysaccharides [a]

| | Yield (mg) from: *E. spinosum* | *E. cottonii* |
|---|---|---|
| 2,3,4,6-Tetra-*O*-methylgalactose [b] | 6 | 58 |
| 2,4,6-Tri-*O*-methylgalactose [b] | 21 | 22 |
| 2,3,6-Tri-*O*-methylgalactose [b] | 15 | 13 |
| 2,6-Di-*O*-methylgalactose | 268 | 285 |
| Mono-*O*-methylgalactoses [b] | 65 | 60 |

[a] Some of the minor components were obtained in mixed fractions, the composition of which was judged visually from paper and gas chromatograms to derive estimates for this Table. [b] The yield of these components might be overestimated, for reasons given in the text.

Although 3,6-anhydrogalactose derivatives are destroyed easily by straightforward hydrolysis or methanolysis, they may be preserved by using an oxidative hydrolysis method.[11,15] The products from such cleavage of both methylated polysaccharides were again characterized rigorously as crystalline materials; the sole anhydride from *E. spinsoum* was thus shown to be 3,6-anhydro-D-galactonic acid, and the sole anhydride from *E. cottonii* was 3,6-anhydro-2-*O*-methyl-D-galactonic acid.

All evidence therefore confirms that these polysaccharides are based on β-1,4- and α-1,3-linked galactose residues with almost all the latter as D-galactose 4-sulphate. In κ-carrageenan from *E. cottonii*, most of the 4-linked residues occur as 3,6-anhydro-D-galactose, with a smaller proportion as D-galactose 6-sulphate. The major difference between the two polysaccharides is that in the ι-carrageenan from *Eucheuma spinosum*, almost all 4-linked residues (whether anhydride or 6-sulphate) carry 2-sulphate, whereas few if any of the corresponding residues in the κ-carrageenan do so. Both polysaccharides evidently have 'masked repeating' [18] structures. After treatment with alkaline borohydride they are more genuinely alternating than any other carrageenans known at this time.

It seems surprising that the two *Eucheuma* species should synthesize polysaccharides that are so clearly distinct. The simplest explanatory hypothesis would be that the biosynthetic routes differ only in there being one additional step in *E. spinosum*, namely a 2-sulphation at some stage before 3,6-anhydride is formed by elimination of 6-sulphate in the usual [19,20] way.

## EXPERIMENTAL

General and chromatographic methods were as described in earlier papers in this series. The solvent for paper chromatography was butan-1-ol–ethanol–water (4 : 1 : 5; upper phase) and the spray was *p*-anisidine hydrochloride, unless stated otherwise.

*Polysaccharide Materials.*—The materials were prepared in the laboratory of Marine Colloids, Inc., Rockland, Maine 04841, U.S.A. as follows. *Eucheuma cottonii* (harvested in the Phillippines) and *Eucheuma spinosum* (harvested in Indonesia) were each air-dried, carefully hand-sorted, and washed free from sand and salts. Extraction was performed with water at 90—95° for 1·5 h. After filtration, the polysaccharide was precipitated by addition of propan-2-ol, then soaked in four changes of aqueous potassium chloride (25% w/v) to convert into the potassium salt. A small amount of the product from *E. spinosum* dissolved at this stage but was discarded since it constituted less than 2% of the total. None of the product from *E. cottonii* dissolved. Each residue was then washed repeatedly with 85% aqueous propan-2-ol until free from salt, and finally dried to a powder. No sub-fractionation could be achieved when a sample of either was dissolved in water and potassium chloride was gradually added. The analyses (Table 1) and i.r. spectra were performed by methods similar to those reported previously.[8] The measurement and calculation of optical rotation for a preparation derived from the *E. spinosum* polysaccharide is described elsewhere.[3,16]

Samples of each polysaccharide were hydrolysed with formic acid (45%; 100° for 16 h) and then with sulphuric acid (0·1N; 100° for 16 h). After neutralization, paper chromatography showed the presence of galactose, and fast-moving products which reacted in the cold with *p*-anisidine hydrochloride spray in the manner characteristic of the degradation products of 3,6-anhydrogalactose.

*Reactions with Alkaline Borohydride and with Periodate.*—The more direct of the two methods described earlier [15] (*i.e.* without prior hydrolysis and reduction) was used. Interpretation of the results in the usual way led to the figures in Table 1, showing good agreement with the results of direct analysis.

*Preparation and Preliminary Examination of the Methylated Polysaccharides.*—Each of the two polysaccharides (10 g) was methylated with sodium hydroxide and dimethyl sulphate.[15,21] Yields were 12·5 (*E. cottonii*) and 11·7 g (*E. spinosum*). Hydrolysis of small samples (45% formic acid at 100° for 16 h) followed by paper chromatography, showed virtually no mono-*O*-methylgalactoses from either

[16] D. A. Rees and F. B. Williamson, to be submitted.
[17] R. A. Jones, E. J. Staples, and A. Penman, *J.C.S. Perkin II*, 1973, 1608.
[18] N. S. Anderson and D. A. Rees, *J. Chem. Soc.*, 1965, 5880.
[19] D. A. Rees, *Biochem. J.*, 1961, **81**, 347.
[20] C. J. Lawson and D. A. Rees, *Nature*, 1970, **227**, 392.
[21] T. C. S. Dolan and D. A. Rees, *J. Chem Soc.*, 1965, 3534.

polysaccharide, and methylation was therefore judged to be essentially complete. This conclusion is confirmed by the more detailed examination of fragmentation products, as described later. Each polysaccharide was converted into the ammonium salt for analysis (Found: OMe, 18·7% for *E. cottonii*; 14·2% for *E. spinosum*).

The paper chromatograms (see before) showed the same pattern of spots from each polysaccharide, assigned as follows: galactose (minute traces), mono-*O*-methylgalactoses (traces), 2,6-di-*O*-methylgalactose (large amount), tri-*O*-methylgalactoses (small amount), tetra-*O*-methylgalactose (traces). Differences were, however, shown between the two products by methanolysis with 2—3% methanolic hydrogen chloride in a sealed tube at 100° for 16 h followed by neutralization with silver carbonate and g.l.c. (polyethylene glycol adipate, 15% on Celite; operated isothermally at 185° in the Pye Argon Chromatograph). The results (Figure) show only small amounts of 3,6-anhydro-2-*O*-methylgalactose derivatives from *E. spinosum* but large amounts from *E. cottonii*.

Control experiments, using the same conditions for methanolysis and g.l.c., showed that methyl 3,6-anhydro-α-D-galactopyranoside [15] was completely decomposed after heating in the sealed tube for 16 h whereas, after the same treatment, methyl 3,6-anhydro-2-*O*-methyl-α-D-galactopyranoside [15] gave a pattern of peaks that could be assigned to glycosides and dimethyl acetal (*cf.* Figure). The former compound could be equilibrated with little decomposition by heating with the methanolic hydrogen chloride under reflux for 1 h but this was of little practical value because complete fragmentation of the polysaccharide is not achieved under these conditions.

*Separation of the Hydrolysis Products of Methylated* Eucheuma *polysaccharides.*—Each methylated polysaccharide (3 g) was hydrolysed in aqueous formic acid (45% at 100° for 16 h). The hydrolysate was evaporated under diminished pressure, with constant addition of water until the distillate was neutral, and then neutralized by titration with barium hydroxide solution, filtered, and evaporated to dryness in a weighed flask; yields 1·57 (*E. cottonii*) and 1·74 g (*E. spinosum*). Part of the product (0·5 g) was dissolved in water and applied to a cellulose column (55 × 4 cm diam.) which was eluted with butanol half-saturated with water, fractions (50 ml) being collected automatically and examined by paper chromatography. Combination of the fractions that had similar compositions, followed by evaporation to dryness and further separation as necessary by paper sheet chromatography [ammonia (*d* 0·880)–water–ethyl methyl ketone (1 : 17 : 200) solvent] gave the yields shown in Table 2. It is likely that minor components are overestimated relative to 2,6-di-*O*-methylgalactose because all fractions include indeterminate amounts of material from the column, from chromatography paper, and from solvent residues, which make a proportionately larger contribution when the weight of sugar is small. The g.l.c. charts (Figure) suggest that the relative amounts of tri-*O*-methyl and tetra-*O*-methyl sugars are very small.

Both polysaccharides gave a 2,6-di-*O*-methylgalactose fraction which crystallized: from *E. cottonii*, m.p. 109—111°, unchanged when mixed with authentic D-enantiomorph, $[\alpha]_D$ +38 ⟶ +83·3° (*c* 0·27 in $H_2O$); from *E. spinosum*, m.p. 110—112°, unchanged when mixed with authentic D-enantiomorph, $[\alpha]_D$ +44·5 ⟶ +89° (*c* 0·15 in $H_2O$).

*Oxidative Hydrolysis of the Methylated Polysaccharides.*—Each methylated polysaccharide (1 g) was dissolved in water (100 ml) and N-sulphuric acid was added, followed by bromine (0·5 ml). The solution was heated at 60° with continuous stirring. After 44 h, the reaction was shown to be complete [15] and the solution was therefore cooled and aerated to remove bromine, before cautious addition of concentrated sulphuric acid to bring the acid concentration to N. After heating at 100° for 16 h and neutralization with barium carbonate, the filtrate was stirred with an excess of silver carbonate for 48 h, filtered again, and passed through a column of Amberlite IR-120 ($H^+$) resin before addition to a column of DEAE-Sephadex (A-25 in the formate form; 20 × 3 cm diam.). Neutral sugars were eluted with distilled water, and then acidic sugars were eluted with aqueous formic acid (2%; 1 l). Each solution was evaporated and the residue was dried ($P_2O_5$) at room temperature; yields 0·16 and 0·39 g for neutral and acid fractions from *E. cottonii*, 0·32 and 0·29 g for neutral and acid fractions from *E. spinosum*.

Paper chromatography [15] showed that each acid fraction consisted essentially of one component, the mobilities of which correspond to 3,6-anhydrogalactonic acid (*E. spinosum*) and its 2-*O*-methyl ether (*E. cottonii*). To confirm these identifications, each fraction was dissolved in methanolic hydrogen chloride (3% w/v) and left at room temperature for 2 h. Esterification was shown to be complete by t.l.c. (silica gel, ethyl methyl ketone as solvent, sulphuric acid spray). After neutralization with silver carbonate and filtration, the solution was evaporated to dryness. The ester from *E. cottonii* was crystallized and recrystallized from benzene to give needles, m.p. and mixed m.p. with authentic methyl 3,6-anhydro-2-*O*-methyl-D-galactonate, 91°; $[\alpha]_D$ +73° (methanol). The ester from *E. spinosum* was characterized by conversion into methyl 3,6-anhydro-D-galactonate 2,4,5-tri-*O*-*p*-nitrobenzoate, m.p. 189—190°, mixed m.p. 187—190°. In earlier work [15] the *p*-nitrobenzoate was purified by preparative t.l.c. but we have now found that an equally pure product is obtained by leaching impurities from the crude product with methanol, followed by crystallization from aqueous acetone by slow evaporation.

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