Carrageenans. Part VIII.[1] Repeating Structures of Galactan Sulphates from *Furcellaria fastigiata, Gigartina canaliculata, Gigartina chamissoi, Gigartina atropurpurea, Ahnfeltia durvillaei, Gymnogongrus furcellatus, Eucheuma cottonii, Eucheuma spinosum, Eucheuma isiforme, Eucheuma uncinatum, Aghardhiella tenera, Pachymenia hymantophora,* and *Gloiopeltis cervicornis*

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In the search for new structures to assist the development of conformational analysis, water-soluble polysaccharides have been extracted from the title algae and where possible separated into 3,6-anhydrogalactose-rich and 3,6-anhydrogalactose-poor components, *i.e.* into κ and λ-fractions. The sixteen polysaccharides were investigated by (i) direct analysis, (ii) measurement of the sulphate elimination to form extra 3,6-anhydrogalactose residues, and (iii) determination of the proportion of the structure that is composed of alternately arranged galactose and 3,6-anhydrogalactose residues, before as well as after the sulphate elimination.

The results were consistent with existing concepts of the structural chemistry of these polysaccharides but several new forms are noted that may be valuable for conformational studies, including an agarose sulphate and several possible new variants of λ-carrageenan.

IMPORTANT new developments in polysaccharide conformation studies [2] have been made possible by the special properties of polysaccharides from algae. The examples that now exist of a polysaccharide double helix,[3] a polysaccharide triple helix,[4] and co-operative order–disorder transitions in polysaccharide solutions [5] including the interaction of unlike polysaccharides in an association that may be termed 'polysaccharide quaternary structure',[6] all involve algal polysaccharides. The galactan suphates of red seaweeds (Rhodophyceae) represent a family of related structures,[7] within which variations can be exploited to show the influence of covalent features on conformational behaviour and physical properties. These insights can be applied to other polysaccharide families as shown by the discovery of a novel double helical structure for hyaluronic acid to account for the elastic properties of important biological fluids,[8] as a direct result of the work on seaweed systems. In this paper we report an investigation of more algal galactans, in the hope of finding further useful members. Examples were chosen for investigation from the results of an earlier and wider survey of analytical composition and physical properties,[9] using physical properties as a convenient guide to conformational behaviour.[10] We now investigate whether the sequence of linkages is as expected for polysaccharides from these sources, and whether certain types of sulphate ester are present. The following paper [11] gives complementary information from methylation analysis.

Measurements, Interpretation, and Assumptions.—The polysaccharides and their sources are listed in the Table with results of the following analyses.

(1) Conventional measurements were made such as specific optical rotation, and the proportions of sulphate ester and of the two types of sugar residue. (The small amounts of other sugars detected in hydrolysates by paper chromatography were attributed to contaminating polysaccharides as discussed elsewhere.[11])

(2) The increase in 3,6-anhydrogalactose residues was measured when the polysaccharide was treated with hot alkaline borohydride, before and after exposure to periodate. Because of previous experience [1,12-15] and the internal consistency of the conclusions that emerge, this increase is interpreted as having arisen from an equimolar amount of galactose sulphate. Other evidence [see (3)] will show that the sulphated residues are linked glycosidially through position 4 and therefore that the periodate-oxidizable members are galactose 6-sulphate.

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[1] Part VII, N. S. Anderson, T. C. S. Dolan, and D. A. Rees, preceding paper.

[2] (*a*) D. A. Rees, *Biochem. J.*, 1972, **126**, 257; (*b*) D. A. Rees, in 'MTP International Review of Science, Organic Chemistry Series One,' 1973, vol 7, p. 251.

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

[4] E. D. T. Atkins and K. D. Parker, *J. Polymer Sci., Part C, Polymer Symposia*, 1969, 69.

[5] A. A. McKinnon, D. A. Rees, and F. B. Williamson, *Chem. Comm.*, 1969, 701; D. A. Rees, W. E. Scott, and F. B. Williamson, *Nature*, 1970, **227**, 390; E. R. Morris, D. A. Rees, and D. Thom, *Chem. Comm.*, 1973, 245; R. A. Jones, E. J. Staples, and A. Penman, *J.C.S. Perkin II*, 1973, 1608; D. A. Rees, and F. B. Williamson, to be submitted.

[6] A. A. McKinnon, I. C. M. Dea, and D. A. Rees, *J. Mol. Biol.*, 1972, **68**, 153.

[7] N. S. Anderson, T. C. S. Dolan, and D. A. Rees, *Nature*, 1965, **205**, 1060.

[8] I. C. M. Dea, R. Moorhouse, D. A. Rees, S. Arnott, J. M. Guss, and E. A. Balazs, *Science*, 1973, **179**, 560.

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

[10] D. A. Rees, *Adv. Carbohydrate Chem. Biochem.*, 1969, **24**, 267.

[11] A. Penman and D. A. Rees, following paper.

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

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

[14] N. S. Anderson, T. C. S. Dolan, and D. A. Rees, *J. Chem. Soc.* (*C*), 1968, 596.

[15] 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.

The remainder are assumed for the moment to be galactose 2,6-disulphate, although other interpretations are possible, including the presence of galactose 3-sulphate and branch points in the polysaccharide structure.

(3) We measured the yield of carrabiose dimethyl acetal from a mild methanolysis [14] which split all 3,6-anhydrogalactosyl bonds but only a small proportion of galactosyl bonds. Through control experiments it was then possible to calculate the yield of carrabiose derivatives which would have been formed in the absence of galactosyl cleavage. When this result is expressed as the fraction of total 3,6-anhydride which can be thus split out as carrabiose derivatives, it is called the ' consecutive carrabiose content '. Since it is the *fraction* of 3,6-anhydride which occurs in available carrabiose residues, a polysaccharide may have a value of 100% for this parameter as here defined but be far from entirely constituted of carrabiose residues (*e.g.* sample 14 after HO^-–BH_4^- in the Table). Similarly, a carrabiose segment (G–A) in a sequence such as G–G–G–G–A–G–G–G (where G is galactose and A is anhydrogalactose) would not contribute to the value because only *consecutive* carrabiose residues are measured. The determination was made before and after treatment with alkaline borohydride, to give information about the distribution of 6-sulphate (and/or 2,6-disulphate) as well as of 3,6-anhydride. Similar measurements have been made for other polysaccharides of the carrageenan family,[14,16] but in a way that is now superseded by the improved method that is described here and is based on g.l.c.

Polysaccharide Types.—On the basis of the measurements just described, polysaccharides are classified as follows.

An agarose sulphate. After modification with alkaline borohydride, the polysaccharide from *Gloiopeltis cervicornis* has roughly equimolar proportions of sulphate ester, galactose, and 3,6-anhydrogalactose residues (Table), suggesting a ' masked repeating structure '.[17] However, the negative optical rotation indicates that it belongs to the agar rather than carrageenan family—and it is the only polysaccharide in the Table of this type. These conclusions were suggested already by high yields of agarobiose derivatives after partial methanolysis of the polysaccharide from a closely related species.[18] It is unusual for an agar structure to have so high a content of alkali-stable sulphate ester, although this is common in the carrageenan series. Further details are revealed by methylation analysis,[11,18] and are discussed elsewhere.[11]

κ-Carrageenans, ι-carrageenans, and hybrids. ' κ-Fractions ' are isolated from whole carrageenans by selective precipitation with potassium chloride.[19] They are expected to have masked repeating structures in which D-galactose 4-sulphate and 3,6-anhydro-D-galactose are arranged alternately but with interruption by the occurrence of galactose 6-sulphate or galactose 2,6-disulphate in place of a proportion of 3,6-anhydride residues.[1,14] These interruptions are called ' kinking residues ' because of their influence on polysaccharide conformation.[20] Some further sulphate often occurs on position 2 of the 3,6-anhydride, and those polysaccharides in which this 2-sulphation is substantially complete are called ι-carrageenans.[1] The three κ-fractions (samples 2—4 in the Table) and three of the whole carrageenans (samples 5—7) conform to this pattern in that, as shown in the Table, they have (i) specific optical rotations in the region +30 to +80°; (ii) 0·4—1·0 sulphate ester groups per monosaccharide residue, (iii) roughly comparable proportions of galactose and 3,6-anhydride before the treatment with alkaline borohydride and equal amounts afterwards, and (iv) ' consecutive carrabiose contents ' close to 100% after the alkaline borohydride treatment.

Analysis of algal polysaccharides

Sample no.	Algal species	Polysaccharide fraction	Source	$[\alpha]_D$ (°)	Relative molar composition galactose : 3,6-anhydride : sulphate	Percentage increase in 3,6-anhydride when treated with alkaline borohydride: Before IO_4^-	Percentage increase in 3,6-anhydride when treated with alkaline borohydride: After IO_4^-	' Consecutive carrabiose contents ': Before HO^-–BH_4^-	' Consecutive carrabiose contents ': After HO^-–BH_4^-	Relative molar composition after HO^-–BH_4^- 3,6-anhydride :galactose
1	*Gloiopeltis cervicornis*	Whole	South Korea, 1967	−30	1·00 : 0·52 : 0·94	21	16			
2	*Furcellaria fastigiata*	κ	Northumberland Strait, Nova Scotia, Summer, 1965	+81	1·00 :0·98 : 0·74	7	3	97	95	1·05
3	*Gigartina canaliculata*	κ	Pacific Coast of Baja, California, 1966	+69	1·00 : 0·64 : 1·20	15	7	83	99	1·01
4	*Gigartina chamissoi*	κ	Peru, 1966	+54	1·00 : 0·82 : 1·31	19	16	69	97	0·99
5	*Eucheuma cottonii*	Whole	← As described elsewhere [1] →					99	96	1·04
6	*Eucheuma spinosum*	Whole	← As described elsewhere [1] →					67	95	1·02
7	*Eucheuma uncinatum*	Whole	Gulf of California, Spring, 1966	+36	1·00 : 0·45 : 1·44	34	23	84	95	0·95
8	*Eucheuma isiforme*	Whole	Florida Keys, 1966	+54	1·00 : 0·56 : 1·45	26	24	85	86	0·99
9	*Ahnfeltia durvillaei*	Whole	Peru, 1966	+44	1·00 : 0·59 : 1·25	18	13	74	84	1·06
10	*Aghardhiella tenera*	Whole	Hungars Creek, Virginia, October, 1964	+67	1·00 : 0·47 : 1·35	23	18	85	88	0·78
11	*Gymnogongrus furcellatus*	Whole	Peru, 1966	+60	1·00 : 0·49 : 1·39	46	25	75	84	0·86
12	*Furcellaria fastigiata*	λ	As for sample 2	+43	1·00 : 0·07 : 0·53	68	7	79	100	0·70
13	*Gigartina canaliculata*	λ	As for sample 3		1·00 : 0·11 : 1·27	89	88	47	65	0·20
14	*Gigartina chamissoi*	λ	As for sample 4	+59	1·00 : 0·16 : 1·17	37	23	35	43	0·48
15	*Gigartina atropurpurea*	Whole	New Zealand, 1965	+59	1·00 : 0·14 : 1·37	100	78	72	100	0·29
16	*Pachymenia himantophora*	Whole	New Zealand, 1965		1·00 : 0·06 : 0·77	50	20	92		

[16] N. S. Anderson and D. A. Rees in ' Proceedings of the Vth International Seaweed Symposium,' eds. E. G. Young and J. L. McLachlan, Pergamon, London, 1966, p. 243.

[17] N. S. Anderson and D. A. Rees, *J. Chem. Soc.*, 1965, 5880.

[18] S. Hirase and K. Watanabe in ' Proceedings of the VIIth International Seaweed Symposium,' University of Tokyo Press, Tokyo, 1973, p. 451.

[19] D. B. Smith, W. H. Cook, and J. L. Neal, *Arch. Biochem. Biophys.*, 1954, **53**, 192.

[20] D. A. Rees, I. W. Steele, and F. B. Williamson, *J. Polymer Sci., Part C, Polymer Symposia*, 1969, 261.

Tentative estimates may be made of the proportion of 3,6-anhydrogalactose 2-sulphate in each polysaccharide if it is assumed that these residues account for the excess of sulphate over galactose. Such estimates suggest that the two *Gigartina* polysaccharides (samples 3 and 4) have the structural features of both κ-carrageenan and ι-carrageenan. When the proportion of ' kinking residues ' exceeds the proportion of 3,6-anhydride, the polysaccharides become soluble in aqueous potassium chloride and are named μ- and ν-carrageenans [9,21] rather than κ- and ι-carrageenans respectively. The polysaccharide from *E. uncinatum* is therefore a hybrid of ι-carrageenan and ν-carrageenan. For none of these polysaccharides is the extent of polydispersity known—except that, in the product from *E. uncinatum*, all the ν-features must be combined in chains which have sufficient ι-features to give insolubility in potassium chloride solution. As already known,[22] the polysaccharide from *Furcellaria fastigiata* contains even less sulphate than a κ-carrageenan and its 3-linked galactose residues must be sulphated incompletely.

In two of the samples (3 and 7 in the Table), the kinking residues are present as both 6-sulphate and 2,6-disulphate, as shown by the effect of periodate on the yield of 3,6-anhydride in the alkaline borohydride reaction. Within experimental error two other samples (3 and, as shown earlier,[1] 6) have entirely 2,6-disulphate in these positions and another two (2 and, as shown earlier,[1] 4) have entirely 6-sulphate. The level of galactose 2,6-disulphate therefore does not correlate so neatly with the level of 3,6-anhydrogalactose 2-sulphate as it did for the samples in the earlier study.[1]

The *distribution* of kinking residues along the chain is shown to vary from one polysaccharide to another by the values for ' consecutive carrabiose content ' before modification with alkaline borohydride. The value may be as high as 99% (sample 5, Table)—indicating that the ' kinks ' here tend to be grouped together, perhaps even in a contaminating polysaccharide—or as low as 67% (sample 6, Table), which shows that most of them are here separated by at least one carrabiose residue.

Deviant ι- and ν-carrageenans. Four more of the whole carrageenans, namely those from *Eucheuma isiforme*, *Ahnfeltia durvillae*, *Aghardhiella tenera*, and *Gymnogongrus furcellatus* (samples 8—11 in the Table), can be assigned to the ι-carrageenan/ν-carrageenan group on the basis of the criteria outlined already. However, a further structural variant is revealed by the failure of the ' consecutive carrabiose content ' to achieve 100% after treatment with alkaline borohydride and, for three of the samples, by the failure of the ratio of galactose to 3,6-anhydride to move to unity after the same reaction. If this is to be explained in terms of a masked repeating structure, it must indicate the presence of some kinking residues that do not carry 6-sulphate and therefore cannot be converted into 3,6-anhydride. Such features are present to a lesser extent in ' normal ' κ- and ι-carrageenans. For one of the samples, this deviation is detected only by the ' consecutive carrabiose content ' and not by the sugar ratio, presumably because the former measurement is more sensitive to the deviation and the experimental method is more accurate. This explanation can only be tentative on the evidence available, and the problem will be re-opened when we present evidence from methylation analysis.[11]

λ-Carrageenans and related polysaccharides. For those polysaccharides that have been isolated by fractionation, we use the usual term ' λ-fraction ' to refer to material that is soluble in aqueous potassium chloride, even though the evidence given in this paper is not sufficient to distinguish between λ-, μ-, and ν-carrageenans. These three types are alike in having a low content of 3,6-anhydride and differ most characteristically in sulphate distribution. The information available suggests that the λ-fractions from *Furcellaria fastigiata*, *Gigartina canaliculata*, and *Gigartina chamissoi* (samples 12—14), and the whole polysaccharides from *Gigartina atropurpurea* and *Pachymenia himantophora* (samples 15 and 16), do each contain material of at least one of these types although they also show features unlike any reported before.

The ' consecutive carrabiose content ' after treatment of the λ-fraction of *F. fastigiata* with alkaline borohydride, shows that large parts of the native structure must contain alternate sequences of non-sulphated galactose and galactose 6-sulphate. However, the sugar ratio after treatment with alkaline borohydride shows that other features must be present, perhaps in a distinct, separate polysaccharide with little sulphate ester.

The three *Gigartina* polysaccharides (samples 13—15) resemble each other but differ from typical [23] λ-carrageenans in having only about 1 sulphate per sugar residue rather than about 1·3, and in giving a low yield of 3,6-anhydride when treated with alkaline borohydride. These and other features of the results, and further evidence concerning the *Pachymenia* polysaccharide, are discussed in the following paper.[11]

EXPERIMENTAL

Isolation of Polysaccharides.—The source of each alga and the analytical data for each polysaccharide are listed in the Table. Therefore it is necessary here to describe only the conditions of extraction and, where applicable, fractionation.

In all extraction procedures described below, 33·3 parts by weight of water or aqueous solution were used for each part of dry alga.

Gloiopeltis cervicornis. This Korean alga was indistinguishable from *Gloiopeltis furcata* from Japanese and other sources. It was air-dried after harvesting for transport to the laboratory, then washed in cold, running water (30 min), drained, and heated (2 h) in boiling water before blending (Waring blendor) and further heating (2 h). After filtration, the polysaccharide was precipitated by addition

[21] N. S. Anderson, T. C. S. Dolan, C. J. Lawson, A. Penman, and D. A. Rees, *Carbohydrate Res.*, 1968, **7**, 468.

[22] T. J. Painter, *Canad. J. Chem.*, 1960, **38**, 112.

[23] T. C. S. Dolan and D. A. Rees, *J. Chem. Soc.*, 1965, 3534.

of propan-2-ol (2—3 vol.), washed (2 vol. 90% v/v water–propan-2-ol), dried at 60°, pulverized, and converted into the potassium salt by stirring the recovered powder at 25° for 12 h in 25 parts by weight of water–propan-2-ol (50% v/v) containing 14% by weight of potassium chloride. The product was recovered on a Buchner filter and treated again three times, using a fresh solution each time, then redissolved in water; the solution was clarified by filtration, and the product reprecipitated with propan-2-ol as before. Finally, it was washed repeatedly with water–propan-2-ol (3 times in 60% v/v and twice in 90% v/v) until the washings gave a negative test for chloride, then dried at 60° and ground to a powder.

Furcellaria fastigiata. The air-dried alga was washed in cold running water (2 h) then extracted with water at 85° (2·5 h) while maintaining the pH between 8 and 8·5. The filtrate was dark in colour. Decolorization could not be achieved with carbon but was possible by addition of calcium chloride followed by sodium silicate and subsequent removal of the flocculent precipitate by filtration. The remaining yellow colour was removed when the product was precipitated by addition of propan-2-ol (2—3 vol) and dried at 60° to a powder.

Fractionation was achieved by leaching out the λ-fraction with aqueous potassium chloride (0·3M). The κ-fraction was dissolved in water and precipitated by addition of potassium chloride (to 0·3M), then redissolved in water. Each solution was clarified by filtration and the polysaccharide product was isolated as already described.

Gigartina canaliculata. The air-dried alga was ion-exchanged by soaking in 10 parts by weight of solution containing 24% by weight of potassium chloride and 1% by weight of potassium hydroxide for 48 h at 20°. The salt solution was then drained and the alga was washed three times with 4 parts of cold tap water for 2 min each time, then stirred in 20 parts by weight of fresh water at 40° for 3 h. The solution of λ-fraction was drained from the residual plant material and clarified by filtration, and polysaccharide was precipitated with propan-2-ol in the usual way. The dry, crude product was leached with aqueous potassium chloride (0·3M) and filtered to remove any traces of κ-carrageenan-like material, and finally isolated in the manner already described for other polysaccharides.

The residues from extraction of the alga with aqueous potassium chloride were extracted further with water (95° for 3 h), and the κ-fraction was precipitated by addition of potassium chloride to 0·6M. This product was dissolved in water and precipitated again with potassium chloride before redissolution and precipitation with propan-2-ol, followed by washing and drying.

Gigartina chamissoi. After washing the air-dried alga in the usual way, the polysaccharide was extracted with water at 90—95° (3 h) with addition of dilute sodium hydroxide from time to time to keep the pH close to 8·0, and then isolated by precipitation with propan-2-ol followed by washing and drying.

To achieve fractionation, the dry powder was soaked in aqueous potassium chloride (0·3M) for 16 h. The residue (largely κ-fraction) was collected by filtration and dissolved in warm water, then cooled and reprecipitated by addition of potassium chloride to 0·3M; it was collected on a filter and redissolved in hot water. To this solution, and to the potassium chloride extract, propan-2-ol was added to precipitate the κ- and λ-fractions separately before washing and drying these products in the usual way.

Eucheuma species. These were extracted as described earlier [1] for *E. cottonii* and *E. spinosum.*

Ahnfeltia durvillaei. This alga was extracted in the manner described for *Gloiopeltis cervicornis.*

Aghardhiella tenera, Gigartina atropurpurea, and *Pachymenia himantophora.* Each of these algae was extracted in the manner described for *Gigartina chamissoi* but no separation was achieved into κ- and λ-fractions.

Gymnogongrus furcellatus. This alga was extracted in the manner described for *Gloiopeltis cervicornis.*

Sugar Residues Present.—Each polysaccharide (10—20 mg) was heated with aqueous formic acid (45%; 2 ml) at 100° for 16 h. Formic acid was removed by evaporation and repeated distillation of water from the residue, in a rotary evaporator. Analysis was performed by paper chromatography [ethyl acetate–pyridine–water (10 : 4 : 3) solvent and *p*-anisidine hydrochloride spray]. Polysaccharide samples 3, 7, and 13 showed galactose only; samples 2, 4, 8, 9, 10, 14, 15, and 16 showed galactose with a trace of xylose; samples 11 and 12 showed galactose with traces of xylose and a sugar that could have been fucose or a mono-*O*-methylgalactose; sample 1 showed galactose and xylose in the approximate ratio 9 : 1, with a trace of fucose or mono-*O*-methylgalactose. (For numbering of polysaccharide samples, see Table.) Most polysaccharides also showed large quantities of sugar degradation products which presumably arose from 3,6-anhydrogalactose residues. Evidence is given elsewhere in this paper for the presence of such residues.

The structural significance of the xylose residues when they occur with these polysaccharides is not known. They seem to be lost during methylation.[11] Perhaps they occur in separate polysaccharides in the skeletal parts of the algal cell walls.

Analytical Methods.—Moisture was determined by drying *in vacuo* at 70° to constant weight. Cations (Na^+, K^+, Ca^{2+}) were determined by flame photometry and compleximetric titration. Total sulphate was determined gravimetrically, following digestion of the sample with nitric acid and destruction of the excess of acid with formaldehyde. Inorganic sulphate was determined gravimetrically, following removal of sulphated polysaccharide by precipitation with a quaternary ammonium salt (Hyamine 1622; Rohm and Haas). Ester sulphate was calculated by difference. Chloride was determined by Mohr titration, following mild hydrolysis with nitric acid. Nitrogen was determined by micro-Kjeldahl digestion. Apparent 3,6-anhydrogalactose was determined by the resorcinol method.[24]

After correction for moisture, inorganic sulphate (as $CaSO_4$ or K_2SO_4), chloride (as KCl), and nitrogenous matter (6 × N), galactose was estimated from apparent 3,6-anhydrogalactose and ester sulphate by the assumption that the polysaccharide contained only these three units. The 3,6-anhydrogalactose estimate was then corrected for interference by galactose, and the calculation was iterated to improve both estimates. Molar ratios were calculated from the corrected values of galactose, 3,6-anhydrogalactose, and ester sulphate thus obtained.

Optical rotations were measured for dilute aqueous solutions (0·1—0·3% with respect to polysaccharide) at 18 ±2°.

Formation of Extra 3,6-Anhydride Residues by Sulphate Elimination with Alkaline Borohydride.—In preliminary experiments to establish suitable conditions for this re-

[24] W. Yaphe and G. P. Arsenault, *Analyt. Biochem.*, 1965, **13**, 143.

action, λ-carrageenan from *Chondrus crispus* [25] and ι-carrageenan from *Eucheuma spinosum* were each (300 ml) dissolved in water (75 ml) with sodium borohydride (150 mg) and the solutions were left for 16—24 h. More sodium borohydride (150 mg) was then added and each solution was divided into four parts. The first was kept for analysis for 3,6-anhydrogalactose residues. Solid sodium hydroxide was added to the other solutions to concentrations, respectively, of 0·01N, 0·1N, and N before heating at 80° and removal of samples (0·10 ml) for analysis [25] for 3,6-anhydrogalactose residues. Thus it was established that suitable conditions for complete reaction were similar for each polysaccharide, namely N-sodium hydroxide at 80° for 9—10 h.

The following standard method of polysaccharide analysis was therefore adopted for the work which is described here. Polysaccharide (20 mg) was dissolved in water (20 ml) with sodium borohydride (20 ml). After 16—24 h at room temperature, more sodium borohydride (20 ml) was added and the solution was cooled in ice before addition of solid (AnalaR) sodium hydroxide to a concentration of N. A part of the solution was removed and neutralized exactly with the calculated quantity of hydrochloric acid. The remainder was heated at 80° for 10 h, then cooled before removal of a sample (10 ml) for neutralization as before. Both neutral solutions were then analysed in triplicate by the resorcinol method.[25] The entire procedure was then repeated to check reproducibility. The results are shown in the Table.

Formation of Extra 3,6-*Anhydride Residues by Sulphate Elimination with Alkaline Borohydride after Periodate Oxidation.*—Each polysaccharide (50 mg) was dissolved in aqueous sodium periodate (0·05M; 20 ml) and kept in the dark at room temperature for 75 h. Excess of periodate was destroyed by ethylene glycol (2 drops), and sodium borohydride (100 mg) was added. After dilution with water (20 ml) and leaving at room temperature for 2 days, the product was isolated by exhaustive dialysis, evaporation, and freeze-drying. The extent of formation of extra 3,6-anhydride residues was then measured as in the preceding experiment.

Carrabiose Dimethyl Acetal.—This compound is used as a reference in experiments described below. It was prepared from commercial furcellaran [from Litex (20 g)] by Painter's method [22] except that the methanolysis products were separated on a cellulose column (60 × 7 cm) [butan-1-ol–ethanol–water (6 : 2 : 1) solvent]. The fractions which contained the desired product were combined and evaporated to a syrup (3·5 g) which was dissolved at 0° in pyridine (70 ml) and acetic anhydride (54 ml). This mixture was heated at 80° for 1 h and poured into iced water. The hexa-acetate was removed on a filter and dissolved in chloroform, then washed in turn with aqueous copper sulphate, aqueous sodium carbonate, and water. The chloroform solution was dried (Na_2SO_4) and evaporated to dryness, and the residue was crystallized from aqueous ethanol. Recrystallization from ethanol gave needles (3·1 g; m.p. 149—150°).

To prepare a standard solution of carrabiose for calibration of the analyses described below, the hexa-acetate (about 20 mg; accurately weighed) was dissolved in redistilled methanol (dry and redistilled; 20 ml) containing 2,2-dimethoxypropane (1 ml). A solution of sodium methoxide was prepared by addition of 2,2-dimethoxypropane (3 ml) to methanol followed after 30 min by bright sodium (20 mg); part of this (1 ml) was added to the hexa-acetate solution and the mixture was left at room temperature (16 h). After neutralization with solid carbon dioxide and evaporation to dryness, deacetylation was shown to be complete by the ^1H n.m.r. spectrum (solvent deuterium oxide).

All the above operations were easily performed quantitatively and the amount of carrabiose in the final dry residue could therefore be calculated.

Analysis of Polysaccharides after Sulphate Elimination.—Each polysaccharide (300 mg) was modified with alkaline borohydride as described above, and isolated by dialysis and freeze-drying. Part of the product was used for analysis for the ' consecutive carrabiose content ' (see below). Another part was dissolved in water (0·4—1·6 mg ml^{-1}) and a solution of carrabiose (see below) was prepared at about the same concentration with respect to carbohydrate residues. Calibration curves were prepared for the phenol–sulphuric assay for carbohydrate [26] using standard solutions of galactose and of methyl 3,6-anhydro-α-D-galactopyranoside, and a calibration curve was prepared with the latter compound only for the resorcinol determination.[27] The concentration of combined 3,6-anhydrogalactose in each solution was then determined directly (resorcinol method), using suitable small samples withdrawn with a micropipette. The galactose content was determined by a difference procedure with the phenol–sulphuric acid assay, in which the contribution by 3,6-anhydrogalactose residues to this assay is calculated from their measured concentration and subtracted from the optical density; this difference is attributed to galactose residues, the concentration of which is read from the calibration curve. The result for each polysaccharide was expressed as the proportion of 3,6-anhydride relative to galactose residues, and then normalised by the factor required to bring this result for carrabiose to unity. The results are given in the last column of the Table. They are completely independent of those given in columns 6 and 7 but cross-comparisons can be made and the agreement is good for most samples. For sample 12, however, there is a bad discrepancy which is not yet explained.

' *Consecutive Carrabiose Content* '.—An aqueous solution was prepared of each polysaccharide, having the 3,6-anhydride concentration between 0·1 and 0·3 mg ml^{-1}. Analysis by the resorcinol method using a calibration curve drawn with carrabiose dimethyl acetal gave the ' apparent carrabiose content '.

A known volume of each solution (equivalent to about 5 mg carrabiose) was withdrawn and freeze-dried in triplicate. The residue was boiled under reflux (30 min) with methanolic hydrogen chloride (0·2%, containing 2,2-dimethoxypropane; 20 ml) then neutralized with silver carbonate. The solution was kept over an excess silver carbonate for at least 20 min before addition of an accurately measured volume (0·250 ml) of a standard solution of sucrose octa-acetate (about 2% w/v). This compound was to serve as the internal standard for g.l.c. After thorough mixing, filtration, and evaporation, the residue was dried *in vacuo*, and dissolved in pyridine–acetic anhydride (1 : 1

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v/v; 5 ml); the solution was heated on a boiling water-bath (1 h) and evaporated to dryness.

The mixture of acetates was dissolved in bis-(2-methoxyethyl) ether (1 ml) for analysis by g.l.c. [SE 52 column (3% on Gas Chrom P) at 225° in a Pye Argon Chromatograph]. Peak areas were measured by triangulation and converted into relative concentrations by reference to a linear calibration graph that had been prepared using known weights of hexa-*O*-acetylcarrabiose dimethyl acetal and sucrose octaacetate.

To obtain the necessary evidence to interpret the results, known weights of carrbiose dimethyl acetal (about 5 mg) were prepared from the hexa-acetate by deacylation, and methanolysed in the same way as the polysaccharide samples. Eventual analysis by g.l.c. showed a recovery of 68·5% of carrabiose derivative, *i.e.* a loss of 31·5% through side-reactions. Each polysaccharide result was therefore corrected on the assumption that this same loss was experienced and expressed relative to the ' apparent carrabiose content ' that had been determined by direct analysis using the resorcinol method (above). It is this final figure that is named the ' consecutive carrabiose content '. It is believed to be accurate to within 5% or better. [For example, the difference between the values for sample 5 before and after treatment with alkaline borohydride (Table) is not believed to be significant.]

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