Carrageenans. Part XI.[1] Mild Oxidative Hydrolysis of κ- and ι-Carrageenans and the Characterisation of Oligosaccharide Sulphates

By **Alistair Penman** † and **David A. Rees,*** † Chemistry Department, The University of Edinburgh, West Mains Road, Edinburgh EH9 3JJ

Special problems are inherent in the unambiguous location of sulphate esters in polysaccharides which contain a high proportion of 3,6-anhydrogalactose, because of the lability of this sugar residue and its glycosidic linkage. We have developed an oxidative hydrolysis of such polysaccharides to give oligosaccharide sulphates in high yield and thus, by separation and characterisation, to establish the sulphate positions. In this way, the presence is confirmed in κ-carrageenan of 3-linked galactose 4-sulphate residues as the major sulphate ester, with smaller amounts of 4-linked 3,6-anhydrogalactose 2-sulphate and 4-linked galactose 6-sulphate.

In the accompanying papers,[2-4] as well as in earlier work by this and other research groups, the location of sulphate esters in polysaccharide structures was often derived from evidence that, strictly, was ambiguous or indirect. For example, methylation analysis cannot alone distinguish between the sites of glycosidic and sulphate substitution and ambiguities in the approach multiply when structures are considered that might be branched. As an additional difficulty, some sulphates may be lost during the methylation step (usually by alkaline elimination) and are therefore undetected by this method. Examples are given in a preceding paper.[2]

More evidence can be obtained if methylation analysis is repeated after a desulphation step which is usually done with cold methanolic hydrogen chloride.[5] This approach was useful in the structure determination of λ-carrageenan,[6] but its value is limited if glycosidic cleavage is extensive during the desulphation step, and such cleavage is unfortunately common, especially for polysaccharides that are rich in 3,6-anhydrogalactose. Since we completed the work to be reported here, an elegant new desulphation reaction has been demonstrated[7] which should overcome this disadvantage and make possible the location of most sulphate esters, apart from those that are labile under methylation conditions.

I.r. spectroscopy[8,9] and stability to alkali[10-12] may

† *Present address*: Unilever Research, Colworth/Welwyn Laboratory, Colworth House, Sharnbrook, Bedford.

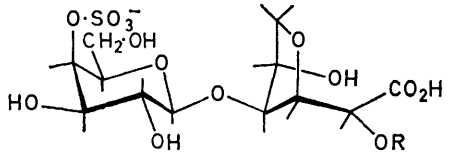
[1] Part X, A. Penman and D. A. Rees, preceding paper.
[2] A. Penman and D. A. Rees, *J.C.S. Perkin I*, 1973, 2182.
[3] C. J. Lawson, D. A. Rees, D. J. Stancioff, and N. F. Stanley, *J.C.S. Perkin I*, 1973, 2177.
[4] N. S. Anderson, T. C. S. Dolan, and D. A. Rees, *J.C.S. Perkin I*, 1973, 2173.
[5] T. G. Kantor and M. Schubert, *J. Amer. Chem. Soc.*, 1957, **79**, 152; R. Johnstone and E. G. V. Percival, *J. Chem. Soc.*, 1950, 1994.
[6] T. C. S. Dolan and D. A. Rees, *J. Chem. Soc.*, 1965, 3534.
[7] A. I. Usov, K. S. Adamyants, L. I. Miroshnikova, A. A. Shapshnikova, and N. K. Kotchetkov, *Carbohydrate Res.*, 1971, **18**, 336.
[8] 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.
[9] D. J. Stancioff and N. F. Stanley, in 'Proceedings of the VIth International Seaweed Symposium,' ed. R. Margalef, Subsecreteria de la Marina Mercante—Direccion General de Pesca Maritima Madrid, 1969, p. 595.
[10] E. G. V. Percival, *Quart Rev.*, 1949, **3**, 369.
[11] D. A. Rees, *J. Chem. Soc.*, 1961, 5168.
[12] D. A. Rees, *J. Chem. Soc.*, 1963, 1821.

also give evidence on sulphate positions. However, the former method depends upon empirical assignments that must be used cautiously because some unexplained anomalies exist,[13] and both methods may fail to reveal types of sulphate that are present in smaller amounts.

The sites of sulphate ester could in principle be characterised by partial fragmentation to sugar sulphates, which are then separated and identified. This has indeed been done for algal galactan sulphates, by both acid hydrolysis [12,14] and enzymic hydrolysis.[15] We now report an attempt to develop another approach of this type for the algal galactan sulphates that are of current conformational interest, namely those which have a high content of 3,6-anhydride residues. The principle is to split anhydrogalactosyl links by the oxidative hydrolysis that was developed for earlier use in methylation analysis,[16] while retaining as much sulphate as possible. This overcomes the problem of side reactions which would follow the exposure of reducing groups of 3,6-anhydrogalactose by simple hydrolysis. The oligosaccharide sulphates are then separated and identified.

Preliminary experiments showed that some degree of sulphate hydrolysis is unavoidable if substantial cleavage to oligosaccharide fragments is to be achieved by oxidative hydrolysis. However, it was possible to establish conditions under which most of the 3,6-anhydrogalactosyl bonds were cleaved whereas much of the sulphate ester remained intact. Few galactosyl bonds are expected to be split under these conditions, and this is confirmed by the nature of the products, as described later.

Hydrolysis of sulphate is more rapid from ι- than from κ-carrageenan, confirming that 2-sulphate is more labile than 4-sulphate as expected from earlier studies.[12,17,18] The products of oxidative hydrolysis of κ-carrageenan were separated by ion-exchange chromatography on diethylaminoethyl-Sephadex, by elution with a concentration gradient of ammonium carbonate. This buffer could readily be removed by evaporation to give several distinct fractions. The major product was carrabionic acid 4-sulphate [*O*-4-sulphato-β-D-galactopyranosyl-(1 ⟶ 4)-3,6-anhydro-D-galactonic acid (I)]. This was



(I) R = H, carrabionic acid 4-sulphate
(II) R = SO_3^-, carrabionic acid 2′,4-disulphate

characterised by methylation analysis, which gave derivatives of 2,3,6-tri-*O*-methylgalactose and 3,6-anhydro-2,5-di-*O*-methylgalactonic acid. That the sulphate was on C(4) was confirmed by its stability to alkali, by the i.r. spectrum, and by Smith degradation to a threitol sulphate.

Carrabionic acid 2′,4-disulphate (II) was also isolated, as expected from the appreciable ι-like character of ' κ-carrageenan ' from *Chondrus crispus*.[16] The amount was small, no doubt because much of the sulphate had been removed from position 2 during oxidative hydrolysis. Identification was based on the following observations. Hydrolysis and paper chromatography showed the presence of galactose and 3,6-anhydrogalactonic acid, but the electrophoretic mobility was greater than that of carrabionic acid 4-sulphate, indicating the presence of an extra charged substituent. Elemental analysis showed an excess of sulphur and the i.r. spectrum showed a peak at 820 cm^{-1} in addition to the 4-sulphate peak at 850 cm^{-1}. Although reference compounds were not available for comparison this extra peak could presumably be attributed to sulphate on position 2 of the open chain 3,6-anhydrogalactonic acid unit. This assignment was confirmed by the resistance of the 3,6-anhydrogalactonic acid residue to periodate oxidation, showing that it was substituted on position 2′ as well as position 4. Treatment with alkali caused the loss of one charged substituent as shown by electrophoresis and as expected on the basis of the structure proposed, with some further rearrangement or decomposition.

An oligosaccharide fraction was also isolated which appeared to contain galactose 6-sulphate residues: warming with alkali caused an increase in its content of 3,6-anhydride. After this treatment the ratio of galactose to 3,6-anhydride was 2 : 1, consistent with a tetrasaccharide structure having an alternating arrangement of galactose 4-sulphate and 3,6-anhydride residues. The structure before alkali treatment would then be (III) or perhaps a sulphate ester of it. In fact, elemental analysis was consistent with structure (III), mixed with a small amount of its product of 6-sulphate elimination which had presumably formed during isolation. Further evidence in support of this structure was as follows. (i) The i.r. spectrum was consistent with the presence of both 4-sulphate (850 cm^{-1}) and 6-sulphate (820 cm^{-1}). (ii) Alkaline elimination of sulphate caused the formation of a stoicheiometric quantity of 3,6-anhydrogalactose but, after periodate oxidation, such 3,6-anhydride formation was not observed. (iii) Methylation analysis gave the derivatives expected, namely those of 2,3,6-tri-*O*-methylgalactose, 2,3-di-*O*-methylgalactose, 2,6-di-*O*-methylgalactose, and 3,6-anhydro-2,5-di-*O*-methylgalactonic acid in approximately equal proportions. (iv) Desulphation [5] followed by methyl-

[13] M. J. Harris and J. R. Turvey, *Carbohydrate Res.*, 1970, **15**, 51.

[14] J. R. Turvey and D. A. Rees, *Nature*, 1961, **189**, 831; T. J. Painter in ' Proceedings of the Vth International Seaweed Symposium,' eds. E. G. Young and J. L. McLachlan, Pergamon, London, 1966, p. 305.

[15] J. Weigl, J. R. Turvey, and W. Yaphe, in ' Proceedings of the Vth International Seaweed Symposium,' ed. E. G. Young and J. L. McLachlan, Pergamon, London, 1966, p. 329; J. R. Turvey and J. Christison, *Biochem. J.*, 1967, **105**, 317.

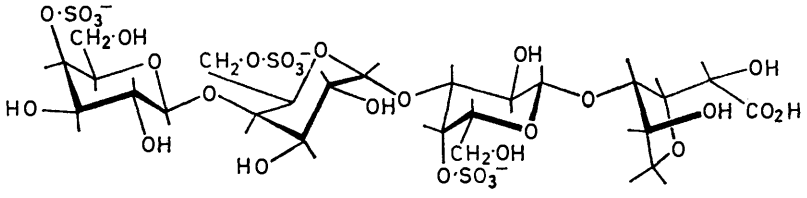
[16] N. S. Anderson, T. C. S. Dolan, and D. A. Rees, *J. Chem. Soc.* (*C*), 1968, 596.

[17] D. A. Rees, *Biochem. J.*, 1963, **88**, 343.

[18] P. F. Lloyd and P. F. Forrester, *Carbohydrate Res.*, 1971, **19**, 430.

ation analysis gave the derivatives expected, namely those of 2,3,4,6-tetra-*O*-methylgalactose, 2,3,6-tri-*O*-methylgalactose, 2,4,6-tri-*O*-methylgalactose, and 3,6-anhydro-2,5-di-*O*-methylgalactonic acid, in approximately equal proportions.

In summary, the main products of mild oxidative hydrolysis of κ-carrageenan are the disaccharide sulphates (I) and (II) and the tetrasaccharide sulphate (III). This provides clear confirmation that the three major sulphate esters are 3-linked galactose-4-sulphate, 4-linked 3,6-anhydrogalactose 2-sulphate, and 4-linked galactose



(III)

6-sulphate. The first of these residues has been widely accepted to be present from the earliest [19] to the most recent [8,15,16] investigations, based on evidence from methylation analysis, i.r. spectra, the stability of this sulphate to alkaline elimination, and the isolation of sugar sulphate fragments. The evidence for the latter two has until now been less conclusive, especially for the 6-sulphate residues which are so important as conformational ' kinks ' which terminate helical parts of the chain.[20]

EXPERIMENTAL

General.—The polysaccharides used for this work were those described earlier in this Series.[4,16] Preparations and analyses of samples by paper chromatography and by g.l.c. were performed by methods described in Part IX.[2] The liquid phase for g.l.c. was always neopentyl glycol adipate (3% on GasChrom P). An additional paper chromatography solvent (n-butanol–ethanol–water, 3 : 1 : 1) was used when the separation involved sugar sulphates, and the spray was then alkaline silver nitrate.[21] For paper electrophoresis, the paper was stretched between two buffer compartments in a closed chamber with no special cooling arrangements. The electrolyte was 0·1M-acetic acid which had been adjusted with pyridine to pH 6·5, and the potential gradient was about 9 V cm^{-1} for up to 2 h. Migration values, M_{GA}, are expressed relative to D-glucuronic acid after allowance for movement caused by endosmosis. The spray was alkaline silver nitrate [21] or aniline–xylose.[22] I.r. spectra were recorded with a Perkin-Elmer 237 spectrometer; the sample was obtained as a film by evaporation on a silver chloride support. Inorganic sulphate was determined by the method of Jones and Letham.[23] Combined 3,6-anhydrogalactose was determined by Yaphe's resorcinol method.[24]

Comparison of Rates for Sulphate Hydrolysis and 3,6-Anhydrogalactosyl Hydrolysis.—Samples (0·35 g) of κ-carrageenan and ι-carrageenan were separately dissolved in water (10 ml). To each was added hydrochloric acid (N; 10 ml) and sufficient liquid bromine to give a small separate layer. Each solution was stirred at 60°, and samples were withdrawn at intervals for determination of residual 3,6-anhydrogalactose residues and of free sulphate, after aeration to remove bromine. After 48 h the solution remaining was aerated and heated at 100° for 16 h to complete the hydrolysis of sulphate ester and so permit the determination of the total amount of sulphate ester that was present at the start of the experiment. The results are shown in the Table.

Carrabionic Acid.—A solution of methanol (25 ml) and 2,2-dimethoxypropane (2·5 ml) was shaken for 1 h, then sodium methoxide solution (2·5 ml; containing 5 mg sodium per ml) was added, followed by hexa-*O*-acetyl-carrabiose dimethyl acetal, prepared as described elsewhere.[3,25] The solution was left for 24 h at room temperature (t.l.c. then showed the deacetylation to be complete) then neutralised with solid carbon dioxide and evaporated to dryness. The residue was dissolved in sulphuric acid (0·5N; 10 ml) and sufficient bromine was added to give a small separate layer. The mixture was heated at 50° until oxidation was complete (resorcinol test on a sample after removal of the bromine by aeration). This required 8 h. After cooling and removal of the bromine by aeration, the solution was neutralised with barium carbonate. Silver carbonate was added to the

Oxidative hydrolysis of carrageenans

	Degree of hydrolysis (%)			
	κ-Carrageenan		ι-Carrageenan	
Reaction period (h)	3,6-Anhydro-galactosyl bonds	Sulphate ester	3,6-Anhydro-galactosyl bonds	Sulphate ester
1·0	37	11	37	*a*
4·0	69	16	62	*a*
6·5	73	20	74	26
11·5	80	20	84	35
24·5	90	38	90	54
48·5	96	59	93	82

[a] Not determined, owing to interference by oligosaccharide sulphates.

[19] J. Buchanan, E. E. Percival, and E. G. V. Percival, *J. Chem. Soc.*, 1943, 51; E. T. Dewar and E. G. V. Percival, *ibid.*, 1947, 1622.

[20] D. A. Rees, I. W. Steele, and F. B. Williamson, *J. Polymer Sci., Part C, Polymer Symposia*, 1969, **28**, 261; C. J. Lawson and D. A. Rees, *Nature*, 1970, **227**, 392.

[21] M. L. Buch, R. Montgomery and W. L. Porter, *Analyt. Chem.*, 1952, **24**, 489.

[22] R. L. Bloch, E. L. Durrum, and G. Zweig, ' A Manual of Paper Chromatography, and Electrophoresis,' 2nd edn., Academic Press, New York, p. 231.

[23] A. S. Jones and D. S. Letham, *Chem. and Ind.*, 1954, 662.

[24] W. Yaphe, *Analyt. Chem.*, 1960, **32**, 1327.

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

suspension, which was left in the dark, with occasional shaking, for 72 h. After filtration and treatment with Amberlite IR 120 (H^+) resin, the solution was concentrated to a syrup.

Oxidative Hydrolysis of κ-Carrageenan and Separation of the Products.—Dried κ-carrageenan (10 g) was dissolved in water (300 ml), and sulphuric acid (1·0N; 300 ml) and bromine (sufficient to give a small separate layer) were added. The mixture was stirred at 60° for 16 h, cooled, aerated to remove bromine, and adjusted to pH 7·5 with saturated barium hydroxide. After centrifugation and adjustment to pH 5·5 with solid carbon dioxide, silver carbonate (30 g) was added and the mixture was stirred in the dark for several days, then filtered and passed through a column of IR 120 (NH_4^+) resin. The solution was concentrated to 500 ml and examined by electrophoresis. The following patterns of spots was observed:

Silver nitrate spray		Aniline–xylose spray	
Mobility	Intensity	Mobility	Intensity
0·00	Weak		
0·53	Weak	0·69	Weak
1·00	++++	1·00	+
1·26	Weak	1·27	+++
1·59	++	1·55	++

The i.r. spectrum showed a strong broad peak near 1240, a strong peak at 850, and a weak peak at 820 cm^{-1}. The hydrolysate (500 ml) was applied to a diethylaminoethyl-Sephadex column in the carbonate form (prepared from 140 g of Sephadex-A50). Elution was performed with a concentration gradient from 0·02M- to 0·3M-ammonium carbonate over 20 l, keeping the pH as low as possible by addition of solid carbon dioxide to the solutions (pH 6·4—7·2). Fractions (50 ml) were collected automatically and analysed by the phenol–sulphuric acid method. Residual material was stripped from the column with 3·0M-ammonium carbonate (5 l). Solutions corresponding to the peaks were examined by electrophoresis and, on the basis of the results, combined into the following fractions:

Fraction A (0·05 g) showed no sulphate ester (i.r.) and no spots appeared on the electrophoretogram when sprayed with aniline–xylose; alkaline silver nitrate showed a neutral spot only.

Fraction B (1·80 g) also showed no sulphate ester i.r. absorption, and aniline–xylose spray revealed components with M_{GA} 0·58 (+) and 1·00 (+++). This fraction was thought to be mainly carrabionic acid, since the mobility of the more intense spot corresponds to this compound.

Fraction C (3·10 g) showed strong sulphate i.r. absorption (1240 and 848 cm^{-1}). Aniline–xylose spray showed a single spot with M_{GA} 1·26. A negligible increase in 3,6-anhydrogalactose content was observed after treatment with alkali. This fraction was therefore thought to be carrabionic acid 4-sulphate.

Fraction D (1·35 g) showed strong sulphate absorption at 1240, 850, and 820 cm^{-1}. Aniline–xylose spray revealed two components in about equal concentrations, with M_{GA} 1·20 and 1·59. Negligible increase in 3,6-anhydrogalactose content was observed after treatment with alkali. This fraction was thought to be a mixture of carrabionic acid 4-sulphate and 2′,4-sulphate.

Fraction E (0·80 g) showed strong sulphate absorption at 1240, 850, and 820 cm^{-1}. Aniline–xylose spray showed spots with mobilities 1·34 (+) and 1·64 (+++). An approximately 30% increase in 3,6-anhydrogalactose content was observed on treatment with alkali.

Fraction F (0·17 g) showed strong sulphate absorption at 1240, 850, and 820 cm^{-1}. Aniline–xylose spray showed spots with M_{GA} 1·36 (+++) and 1·64 (+). An approximately 160% increase in 3,6-anhydrogalactose content was obtained on treatment with alkali. Fractions E and F were thought to contain 6-sulphated fragment(s).

Fraction G (0·28 g) showed sulphate ester absorption at 1240 cm^{-1} but the 800—850 cm^{-1} region was too diffuse to allow any definite conclusions to be drawn. No spots were observed with aniline–xylose spray, and an approximately 420% increase in 3,6-anhydrogalactose content was obtained on treatment with alkali. This fraction was thought to contain 6-sulphated material of high molecular weight.

Characterisation of Carrabionic Acid 4-Sulphate.—The identification of Fraction C as carrabionic acid 4-sulphate is supported by the following evidence. A weighed amount (0·211 g) was made up to 10 ml with water. Phenol–sulphuric acid determination [26] with reference to a calibration curve showed that the galactose concentration was 78·6 μg in 0·01 ml. This would fit a carrabionic acid 4-sulphate sample of 93·9% purity (Found: C, 29·25; H, 6·05; N, 5·65; S, 6·75. Calc. for $C_{12}H_{26}N_2O_{14}S$: C, 31·7; H, 5·75; N, 6·15; S, 7·05—or, with 6% water—C, 29·8; H, 6·05; N, 5·8; S, 6·6%), $[\alpha]_D$ +15·3 (*c* 1·98 in H_2O, this concentration being based on the phenol–sulphuric acid determination). Fraction C (10 mg) in water (1 ml) and saturated aqueous barium hydroxide (2 ml) were heated on a water-bath at 80° for 3 h. The solution was then cooled and neutralised with dilute sulphuric acid. After removal of barium sulphate by centrifugation, electrophoresis showed only starting material. This result is consistent with sulphate ester on position 4 of carrabionic acid.

Fraction C (100 mg) in water (10 ml) was stirred vigorously while dimethyl sulphate (6 ml) and sodium hydroxide solution (30% v/v; 18 ml) were added dropwise and simultaneously over 6 h. This treatment was repeated twice, then the solution was made alkaline and applied to a charcoal–Celite column (24 × 2·2 cm), which was eluted with water. Most of the inorganic ions were eluted before the carbohydrate product which was then isolated by evaporation and freeze-drying. A small portion was hydrolysed by heating in 2N-sulphuric acid on a boiling water-bath for 16 h. After neutralisation with barium hydroxide and treatment of the filtrate with Amberlite IR 120 (H^+) resin, paper chromatography showed 2,3,6-tri-*O*-methyl-galactose and 3,6-anhydro-2,5-di-*O*-methyl-galactonic acid as the only detectable products. An authentic sample of the latter was kindly supplied by Professor Choji Araki and was clearly distinguishable from 3,6-anhydro-2,4-di-*O*-methylgalactonic acid.

A further sample of the methylated product was treated with methanolic hydrogen chloride (3% v/v; 100° for 16 h) for analysis by g.l.c., which showed 2,3,6-tri-*O*-methylgalactosides [t_R 2·15 (strong), 2·64 (weak), 2·89 (medium)], and a peak that was presumed to arise from the methyl ester of 3,6-anhydro-2,5-di-*O*-methylgalactonic acid (t_R 5·46).

A Smith degradation [27] of carrabionic acid 4-sulphate should yield threitol 2-sulphate as the only charged product.

[26] M. Dubois, K. A. Gilles, J. K. Hamilton, P. A. Rebers, and F. Smith, *Analyt Chem.*, 1956, **28**, 350.

[27] I. J. Goldstein, G. W. Hay, B. A. Lewis, and F. Smith, *Methods Carbohydrate Chem.*, 1965, **5**, 361.

Periodic acid (0·3 g) in water (5 ml) and Fraction C (100 mg) were left together in the dark at room temperature for 24 h before addition of aqueous barium hydroxide to precipitate inorganic ions which were removed by centrifugation. Potassium borohydride (100 mg) was added and the solution was left at room temperature for 24 h then acidified with dilute sulphuric acid. Excess of acid was added (to 1·0N) and after a further 24 h at room temperature the solution was neutralised with barium hydroxide, centrifuged, and treated with Amberlite IR 120 (H^+) resin. It was applied to a column of diethylaminoethyl-Sephadex in the carbonate form (prepared from 10 g of A25), which was then eluted with water (500 ml) followed by 0·5M-ammonium carbonate (pH 8·2; 300 ml). The fraction eluted with water showed a strong, electrophoretically immobile spot with traces of a spot having M_{GA} 1·05. The fraction eluted with ammonium carbonate showed an immobile spot (+), and a spot having M_{GA} 1·05 (++). Paper chromatography of the latter fraction showed a spot with the same mobility as threitol (+) and a component with $R_{threitol}$ 0·43 (++). After an attempt to purify the latter by electrophoresis on thick paper, paper chromatography showed spots having $R_{threitol}$ 0·29, 0·62, and 1·24. Subsequent hydrolysis with 45% formic acid at 100° for 16 h followed by rechromatography gave threitol (clearly distinguished from erythritol) and a second spot having $R_{threitol}$ 1·38. The latter is probably formyl ester: authentic threitol gave the same two spots when similarly treated with formic acid. The properties of the material eluted with ammonium carbonate are therefore consistent with a threitol sulphate: its contamination with threitol in the column fractions is explicable in terms of alkaline elimination at the pH of the ammonium carbonate, and the several spots observed after electrophoretic isolation probably correspond to different salt forms and/or anhydrides.

Characterisation of Carrabionic Acid 2′,4-Disulphate.—The i.r. spectrum and electrophoretic behaviour of Fraction D suggest a mixture of carrabionic acid 4-sulphate and 2′,4-disulphate. In an attempt to obtain the pure disulphate, Fraction D (1·20 g) was dissolved in ammonium carbonate (0·02M; 50 ml) and applied to a column of diethylaminoethyl-Sephadex in the carbonate form (prepared from 140 g of Sephadex-A50). Elution was performed with a concentration gradient from 0·02M to 0·15M over 20 l. Fractions (50 ml) were collected automatically and analysed by the phenol–sulphuric acid method; selected fractions were examined by electrophoresis then combined to give carrabionic acid 4-sulphate (0·62 g), a mixture of carrabionic acid 4-sulphate and 2′,4-disulphate (0·15 g), and pure carrabionic acid 2′,4-disulphate (0·19 g). The *disulphate* was characterised as follows. Electrophoresis showed one spot only with M_{GA} 1·55. The i.r. spectrum showed a broad strong peak at 1240 with peaks of approximately equal intensity at 850 and 820 cm^{-1} (Found: C, 24·8; H, 6·45; N, 8·2; S, 9·25. $C_{12}H_{29}N_3O_{17}S_2$ requires C, 26·1; H, 5·25; N, 7·6; S, 11·6%). Hydrolysis in 2N-sulphuric acid on a boiling water-bath for 16 h then neutralisation with barium hydroxide and treatment of the filtrate with Amberlite IR 120 (H^+) resin gave galactose and 3,6-anhydrogalactonic acid as the only products detectable by paper chromatography. Periodic acid (0·2 g) in water (5 ml) and the purified disulphate (30 mg) were left together in the dark for 24 h. The mixture was then neutralised with aqueous barium hydroxide and centrifuged, and sulphuric acid (4N) was added (to a final concentration of 2N). The solution was heated and deionised as before, after which paper chromatography showed no galactose but 3,6-anhydrogalactonic acid as the only sugar product. Finally, the purified disulphate (10 mg) in water (1 ml) was heated with saturated aqueous barium hydroxide (2 ml) on a water-bath at 80° for 3 h. The solution was cooled and neutralised with dilute sulphuric acid, after which electrophoresis showed spots with M_{GA} 0·00 (++), 0·75 (+), and 1·00 (+++).

Characterisation of Galactose 6-Sulphate-containing Tetrasaccharide.—From the foregoing preliminary experiments, Fractions E—G were thought to contain galactose 6-sulphate residues. The galactose : 3,6-anhydride ratios for these fractions were measured after treatment with alkali, as follows.[28] Part of each fraction (10 mg), in water (5 ml), was mixed with sodium hydroxide (2N; 5 ml) and heated at 80° for 3 h. The proportions of galactose and 3,6-anhydrogalactose residues were then determined by reference to calibration graphs prepared for the phenol–sulphuric acid determinations using standard solutions of galactose and methyl 3,6-anhydro-α-D-galactoside, and for the resorcinol determinations using the latter compound only. The concentration of combined 3,6-anhydrogalactose in each solution was determined directly (resorcinol method), and the galactose content was determined by difference (by subtraction of the calculated contribution of 3,6-anhydrogalactose from the total optical density in the phenol–sulphuric acid determination, and reading this difference off the calibration curve). A check of this procedure by analysis of carrabiose dimethyl acetal gave replicate values of 1·02 and 1·05 for the galactose : anhydride ratio. After alkaline modification of Fractions E—G, the values found for this ratio were, respectively, 3·13, 1·99, and 2·30. Considered by themselves these results would be consistent with the presence of the tetrasaccharide (III) in Fractions E and F with contamination by carrabionic acid derivative(s) in the former. However, the evidence (already cited) that 3,6-anhydrogalactose is present before alkaline modification would indicate that both Fractions are contaminated with other oligosaccharides, perhaps by the 6-sulphate elimination product of (III). Fraction G probably contains the tetrasaccharide (III) with higher oligosaccharides. All three Fractions were therefore combined for rechromatography in an attempt to isolate (III) in pure form for characterisation. The combined material in 0·1M-ammonium carbonate (50 ml) was applied to a diethylaminoethyl-Sephadex column in the carbonate form (prepared from 140 g of Sephadex-A50) and eluted with a linear gradient from 0·1M- to 0·3M-ammonium carbonate over 10 l (pH 7·2—8·1). Fractions (50 ml) were collected automatically. Analysis by the phenol–sulphuric acid method showed two main peaks, the second of which was shown by the usual alkaline elimination measurements to contain the higher proportion of 6-sulphate residues. Its i.r. spectrum showed $\nu_{max.}$ 1240, 850, and 820 cm^{-1} and its analytical figures were N, 5·35; S, 8·05% (Calc. for $C_{24}H_{52}N_4O_{30}S_3$: N, 5·75; S, 9·9. Calc. for $C_{24}H_{47}N_3O_{26}S_2$: N, 4·9; S, 7·45%). These calculated values correspond to (III) and its 6-sulphate elimination product respectively, in the ammonium salt forms. Since the experimental figures lie between these two possibilities it is possible that the two tetrasaccharide sulphates are separating on the column but

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

that elimination of 6-sulphate occurs during subsequent isolation. A sample (40 mg) was dissolved in sodium hydroxide (0·1N; 7 ml) and heated at 80° on a water-bath; portions were withdrawn from time to time for 3,6-anhydrogalactose analysis by the resorcinol method, and after exact neutralisation, with the calculated amount of 0·1N-hydrochloric acid, for sulphate analysis with chloroaminobiphenyl.[23] (Preliminary experiments showed no interference with sulphate determination by NH_4^+ or Cl^- at these concentrations.) The results for sulphate release (in μg ml^{-1}), compared with values expected from the measured increase in 3,6-anhydride (shown in parentheses) were: 2 h, 65 (68); 4 h, 127 (122); 8 h, 318 (230); 24 h, 908 (586). Evidently there is a stoicheiometric relation between sulphate release and anhydride formation at early stages: then follows a more comprehensive alkaline degradation leading to an excess of sulphate release.

A further sample (5 mg) was dissolved in water (5 ml) and samples (1·00 ml) were withdrawn for the following determinations.

(i) To one such sample was added periodic acid (0·02M; 1·00 ml) and the solution was left in the dark at room temperature for 48 h. The excess of periodate was precipitated by addition of saturated aqueous barium hydroxide (3·00 ml) and, after centrifugation, a portion (3·00 ml) was withdrawn, mixed with sodium hydroxide (4N; 1·00 ml), and heated at 80° for 3 h. Samples (1·00 ml) were removed for analysis of 3,6-anhydrogalactose content.

(ii) Another sample (1·00 ml) was added to a mixture of periodic acid (0·2M; 1·00 ml) and saturated aqueous barium hydroxide (3·00 ml). The solution was centrifuged and a portion (3·00 ml) was withdrawn, mixed with sodium hydroxide (4N; 1·00 ml) and heated at 80° as before. Samples (1·00 ml) were removed for analysis.

(iii) A third sample was treated in the same way as the second except that water (1·00 ml) was used in place of 4N-sodium hydroxide. On the basis of assumptions outlined in the Discussion section, the differences between the results of the first and third determinations is proportional to the amount of galactose 2,6-disulphate, whereas the difference between the second and third is proportional to the total amount of galactose 6-sulphate. It could therefore be shown that no more than 10% of the 6-sulphated residues were sulphated additionally on position 2.

A further sample of the purified tetrasaccharide (40 mg) in water (10 ml) was methylated as described for carrabionic acid 4-sulphate. After elution from the charcoal–Celite column, hydrolysis of a small part showed that the product was undermethylated and the methylation and desalting procedure was therefore repeated. A part of the final product was hydrolysed (2N-sulphuric acid on a boiling water-bath for 16 h); the product was neutralised with barium hydroxide, and the filtrate was treated with Amberlite IR 120 (H^+) resin. Paper chromatography showed the presence of 2,3,6-tri-*O*-methylgalactose, 2,6- and 2,3-di-*O*-methylgalactoses, and 3,6-anhydro-2,5-di-*O*-methylgalactonic acid, all in approximately equal amounts. A further sample of the methylated material was methanolysed for g.l.c., which showed methyl 2,3,6-tri-*O*-methylgalactosides (t_R 2·09, 2·62, 2·88), methyl 2,6-di-*O*-methylgalactosides (t_R 5·48, 6·44, 7·15, 8·51), methyl 2,3-di-*O*-methylgalactosides (t_R 5·48, 6·44, 7·45), and the methyl ester of 3,6-anhydro-2,5-di-*O*-methylgalactonic acid (τ_R 5·48).

A further part of the purified tetrasaccharide (15 mg), in methanolic hydrogen chloride (0·8%; 10 ml), was stirred for 16 h then neutralised with diazomethane and evaporated to dryness. Electrophoresis showed a neutral spot with no electrophoretically mobile material and therefore that desulphation was complete. The product was then methylated by shaking with dimethylformamide (5 ml), silver oxide (1·5 g), and methyl iodide (10 ml) for 5 days. After filtration and dilution with a large volume of methanol, silver salts were removed by centrifugation and the product was isolated by evaporation. Examination by hydrolysis and paper chromatography showed 2,3,4,6-tetra-*O*-methyl-, 2,3,6-tri-*O*-methyl-, and 2,4,6-tri-*O*-methyl-galactoses and 3,6-anhydro-2,5-di-*O*-methylgalactonic acid, all in approximately equal concentrations. Methanolysis and g.l.c. showed tetra-*O*-methylgalactosides (t_R 1·48; strong), 2,3,6-tri-*O*-methylgalactosides (t_R 2·20, 2·74, 2·94; strong), 2,4,6-tri-*O*-methylgalactosides (t_R 2·74, 2·94; strong), 3,6-anhydro-2-*O*-methylgalactose derivatives (t_R 4·19, 4·64; weak), and methyl ester of 3,6-anhydro-2,5-di-*O*-methylgalactonic acid (t_R 5·46; strong).

We thank Marine Colloids, Inc., for a research grant, for polysaccharide materials, and for discussion with their research staff.

[3/529 *Received*, 12*th March*. 1973]