649. *The Carrageenans. Part II.*[1] *The Positions of the Glycosidic Linkages and Sulphate Esters in λ-Carrageenan*

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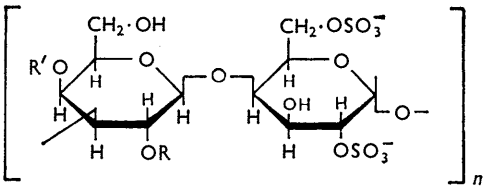
The major structural units present in a preparation of λ-carrageenan have been characterised by methylation analysis before and after removal of the sulphate ester groups. In this way, the polysaccharide has been shown to contain approximately equal proportions of 1,4- and 1,3-linked galactose units. The 1,4-linked units occur as the 2,6-disulphate, whereas the 1,3-linked units are occasionally non-esterified but more frequently occur as the 2-sulphate and relatively rarely as the 4-sulphate. The definition of λ-carrageenan is discussed.

Although it is over ten years since it was shown [2] that carrageenan could be separated into two fairly distinct components termed κ- and λ-carrageenan, the chemical structure of the λ-component has until recently been very poorly understood. One of the glycosidic linkages present was characterised by the isolation of 3-*O*-α-D-galactopyranosyl-D-galactose after acetolysis of λ-carrageenan,[3] although it is not clear to what extent the isolation of this disaccharide can be held to establish the configuration of the glycosidic linkage, in view of the known fact that the acetolysis of some glycosides is preceded by rapid anomerisation.[4] At the same time, a trisaccharide was isolated which was thought to be *O*-α-D-galactopyranosyl-(1→3)-*O*-α-D-galactopyranosyl-(1→3)-D-galactose, suggesting that some contiguous 1,3-linkages occurred. The evidence for the structure of this trisaccharide was not conclusive however, and indeed the structure proposed does not account convincingly for the periodate oxidation data or the optical rotation properties. We consider that until the structure is definitely proven, the isolation of this trisaccharide should not be taken into account in deducing the structure of λ-carrageenan. In Part I we showed that β-1,4 linkages were also present in λ-carrageenan and moreover that 40—50% of the structural units consist of 4-linked galactose 2,6-disulphate. We now report further structural studies and show that the accumulated evidence is consistent with a structure (I)

(I) R varies from unit to unit, but is usually SO_3^-, and less frequently H.

When R = SO_3^-, R′ = H.
When R = H, R′ is usually H, and less frequently SO_3^-.

(II) R varies from unit to unit, but is usually SO_3^-, and less frequently H. R′ = H.



for λ-carrageenan, in which equal proportions of 1,3-linked and 1,4-linked galactose units are present. The 1,4-linked units occur as the 2,6 disulphate, whereas the 1,3-linked units are occasionally non-esterified but more frequently occur as the 2-sulphate and relatively rarely as the 4-sulphate. Several qualifications must be made, however. The structure (I) is shown as a repeating one because this is the simplest of the alternatives, not because an alternating sequence of 1,3- and 1,4-linkages has been conclusively proven. Nevertheless, at least some regions of alternating structure do exist,[1] and the fact (see below) that approximately equal proportions of 1,3- and 1,4-linkages are present is suggestive of an alternating structure. It is also relevant at this point to reconsider the definition of λ-carrageenan. The term was originally used [2] to designate the polysaccharide material which remained in the supernatant solution after selective precipitation of the κ-component

[1] Part I, D. A. Rees, *J.*, 1963, 1821.
[2] D. B. Smith, W. H. Cook, and J. L. Neal, *Arch. Biochem. Biophys.*, 1954, **53**, 192.
[3] K. Morgan and A. N. O'Neill, *Canad. J. Chem.*, 1959, **37**, 1201.
[4] B. Lindberg, *Acta Chem. Scand.*, 1949, **3**, 1153.

with potassium chloride. κ-Carrageenan consists very largely, if not entirely,[5,6] of a linear alternating sequence of galactose 4-sulphate and 3,6-anhydrogalactose units. In contrast, λ-preparations are of varying composition. A survey of a number of samples has shown [6,7] that the structural features already listed (I) are normally present, together with varying amounts of 3,6-anhydrogalactose. In some samples, the concentration of 3,6-anhydrogalactose is low (less than 1%), for example, in the sample used in the present study. In such cases, the concentration of 4-sulphate is also low. These two structural features are therefore more characteristic of κ-carrageenan than λ-carrageenan, and it seems likely that when they do occur in λ-preparations they arise by contamination, or modification of a more fundamental structure. Any such modification might well be a biological phenomenon, in view of the possibility [1] that λ-carageenan is a precursor of κ-carrageenan. We therefore propose to *define* λ-carrageenan as a molecule (II) devoid of 4-sulphate and 3,6-anhydrogalactose, *i.e.*, as one extreme of the spectrum of varying composition. Whether the λ-preparations containing appreciable proportions of 3,6-anhydrogalactose and galactose 4-sulphate units are mixtures of κ-carrageenan and λ-carrageenan, or whether they are to some extent structural hybrids of the two species, has yet to be determined and is under active investigation in this laboratory.*

The positions of the glycosidic linkages were deduced by methylation analysis of desulphated λ-carrageenan. λ-Carrageenan was somewhat resistant to desulphation with hydrogen chloride in methanol,[8] but by repeated treatment under rather forcing conditions, virtually all the sulphate was removed. It is interesting that the infrared spectra (cf. Part I) of samples taken at intermediate stages of desulphation, showed the rather rapid removal of equatorial sulphate ester followed by the slower removal of sulphate esters of axial and primary hydroxyl groups, in agreement with a recent generalisation [9] regarding the relative stabilities of these various types under acid conditions. Methylation of the desulphated polysaccharide was successfully accomplished in a single stage by Kuhn and Trischmann's method,[10] and after hydrolysis the products were separated by gradient elution from a charcoal column. The yield of 2,3,4,6-tetra-*O*-methyl-D-galactose corresponded to the cleavage during desulphation of about one glycosidic bond in every seven of the original polysaccharide. Two tri-*O*-methyl-D-galactoses were isolated in similar yields and identified by conversion into crystalline derivatives. These were 2,4,6-tri-*O*-methyl-D-galactose [this providing additional proof for the presence of the 1,3-linkage shown in (I)], and 2,3,6-tri-*O*-methyl-D-galactose, which could have arisen from 1,4- and/or 1,5-linked galactose units. The 1,5-linkage is considered unlikely however, because all these units were found (see below) to be converted into 3,6-anhydrogalactose units by alkaline elimination of 6-sulphate. Since the formation of a 3,6-anhydro-ring in a galactofuranose unit is sterically impossible, the units cannot be 1,5-linked, and the 2,3,6-tri-*O*-methyl-D-galactose must have arisen from 1,4-linked galactose. No indication for the presence of any other linkages (or of branching) was obtained from the methylation experiments.

In order to obtain evidence for the location of the sulphate ester, λ-carrageenan was methylated with the sulphate groups intact. That some elimination of 6-sulphate occurred under the alkaline conditions of methylation was shown by analysis for 3,6-anhydrogalactose before and after methylation. However, only about 15% of the 6-sulphate was

* *Note Added in Proof.*—Since this manuscript was submitted, we have succeeded in separating alkali-modified λ-carrageenan into two fractions, one of which (11% yield) has been shown by methylation analysis to contain all the galactose 4-sulphate units. This finding supports the view that λ-carrageenan as defined by formula (II) is a distinct chemical entity, and that "λ-preparations" are often mixtures of λ-carrageenan (II) and other material.

[5] A. N. O'Neill, *J. Amer. Chem. Soc.*, 1955, **77**, 6324.
[6] N. S. Anderson, T. C. S. Dolan, and D. A. Rees, *Nature*, 1965, **205**, 1060, and unpublished results.
[7] W. A. P. Black, W. R. Blakemore, J. A. Colquhoun, and E. T. Dewar, unpublished results.
[8] R. Johnstone and E. G. V. Percival, *J.*, 1950, 1994.
[9] D. A. Rees, *Biochem. J.*, 1963, **88**, 343.
[10] R. Kuhn and H. Trischmann, *Chem. Ber.*, 1963, **96**, 284.

affected in this way. The major product of hydrolysis of the methylated polysaccharide was 3-*O*-methyl-D-galactose which was isolated as the crystalline sugar. If it is assumed that the units that gave rise to the 3-*O*-methyl-D-galactose were all of the same type, it follows from the methylation evidence for the desulphated polysaccharide that these are linked glycosidically through position 4 and sulphated on positions 2 and 6. Very strong evidence for the presence of such 1,4-linked galactose 2,6-disulphate units has in fact been obtained by other methods,[1] and the yield of 3-*O*-methyl-D-galactose, especially when correction is made for the proportion of 6-sulphate that underwent elimination during methylation, is in agreement with the estimated proportion of these units, *i.e.*, 45—58% of the structural units in this sample of λ-carrageenan. Further confirmation that the 3-*O*-methyl-D-galactose arose from such 6-sulphated units was obtained in the present study by showing that if all the 6-sulphate is eliminated by treatment of λ-carrageenan with alkali, no 3-*O*-methyl-D-galactose can be detected after subsequent methylation and hydrolysis.

The remaining methyl ethers that were isolated and characterised were 4,6-di-*O*-methyl-D-galactose (31%), 2,4,6-tri-*O*-methyl-D-galactose (13%) and 2,6-di-*O*-methyl-D-galactose (8%). The 2,6-di-*O*-methyl-D-galactose presumably arose from 1,3-linked 4-sulphate rather than 1,4-linked 3-sulphate units because this di-*O*-methyl sugar was also present in the hydrolysate of methylated alkali-treated λ-carrageenan. Such 1,3-linked 4-sulphate units are not included in the structure (II) suggested for λ-carrageenan, for reasons already outlined. The interpretation of the isolation of the 4,6-di-*O*-methyl and 2,4,6-tri-*O*-methyl ethers is quite unambiguous; both of these are derived from the 1,3-linked units that were shown to be present by methylation analysis of the desulphated polysaccharide. The presence of the 1,3-linked galactose 2-sulphate units which is clearly shown by the isolation of the 4,6-di-*O*-methyl-D-galactose, has not previously been suspected in λ-carrageenan.

Experimental

General and Analytical Methods.—See Part I and references therein. For paper chromatography the solvents used were (*A*) butanol–ethanol–water (4 : 1 : 5, upper phase) and (*B*) ethyl acetate–acetic acid–formic acid–water (18 : 3 : 1 : 4), and the spray was *p*-anisidine hydrochloride in butanol.[11] Gas–liquid chromatography of methylated sugars as their equilibrium mixtures of glycosides was carried out using essentially the same apparatus and techniques as described by Aspinall.[12] Thin-layer chromatography was carried out on microscope slides (N. G. Richardson, unpublished method) coated with Silica Gel G containing calcium sulphate as binder, with benzene–ethanol mixtures as solvents, and anisaldehyde–sulphuric acid spray.[13] Paper electrophoresis was carried out using Bouveng and Lindberg's method,[14] with 0·1M-borate buffer (pH 10) and a potential gradient of 30 volts/cm. for 75 min. The paper was clamped between two cooling plates at a pressure of 20 lb/sq. in. For charcoal column chromatography, May and Baker's " Decolourising " grade was used.

λ-*Carrageenan.*—The material was a different batch from that used in Part I. It was kindly prepared for us from *Chondrus crispus* by Marine Colloids Inc., of Rockland, Maine, U.S.A. It had 50·0% galactose (anhydro basis), 0·42% 3,6-anhydrogalactose, and 31·1% sulphate (as SO_3). When it was treated with alkali–borohydride according to the procedure given in Part I, 45% of the galactose units were converted into 3-6-anhydrogalactose with the release of 1·30 molar equivs. of sulphate. For reasons given in Part I, the proportion of 6-sulphated units present as calculated from the 3,6-anhydride formed (45%) is perhaps an underestimate, whereas the figure calculated from the sulphate released (58%) is an overestimate. From these analyses it can therefore be concluded that 45—58% of the galactose units in the

[11] L. Hough, J. K. N. Jones, and W. H. Wadman, *J.*, 1950, 1702.
[12] G. O. Aspinall, *J.*, 1963, 1676.
[13] E. Stahl and U. Kaltenback, *J. Chromatog.*, 1961, **5**, 351.
[14] H. Bouveng and B. Lindberg, *Acta Chem. Scand.*, 1956, **10**, 1283.

present sample are 6-sulphated. $[\alpha]_D$ was +74° in water. Acid hydrolysis followed by paper chromatography showed the presence of galactose only, with traces of xylose and fucose. In the ultracentrifuge, this sample of λ-carrageenan gave a single sharp peak which was distinct from κ-carrageenan.

Desulphation of λ-Carrageenan.—The polysaccharide was dried over phosphoric oxide *in vacuo* at 60° (18·7 g.) and then suspended in methanol (1·8 l.) containing acetyl chloride (27 ml.) and stirred on a heated stage for 48 hr. so that the temperature of the mixture remained at 35°. The polysaccharide was filtered off, washed with methanolic ammonia (1%) and then methanol, and dried *in vacuo* at room temperature and then at 60°. After the process had been repeated a further three times the sulphate content was reduced to the required figure (Found: 91% galactose, 2·0% sulphate; Yield 60% expressed in terms of galactose recovery).

Methylation of Desulphated λ-Carrageenan.—The procedure is a modication of Kuhn and Trischmann's method.[10] The conditions were found important, and are therefore described in full. Polysaccharide (3·0 g.) was added to dimethyl sulphoxide (50 ml.) in a 250 ml. round-bottomed flask with a ground-glass neck, and heated over a flame until a clear solution was obtained. This was cooled under the tap and dimethylformamide (50 ml.) was added. After stirring in ice–water for 30 min., barium hydroxide octahydrate (AnalaR, 50 g.) was added and the mixture stirred efficiently in a closed system for a further 30 min. To the thick white slurry was then added the first portion (9 ml.) of dimethyl sulphate, and the stirring in ice was continued. Further portions of dimethyl sulphate (9 ml.) were added 1, 1½, and 2 hr. later; stirring at 0° was continued for a further 30 min. and then the ice-bath was removed. The reaction was exothermic, the maximum temperature (about 50°) being reached approximately 1 hr. after removal of the bath. The mixture was stirred in a closed system at room temperature for 3 days, then concentrated ammonia solution (20 ml.) was added, followed by stirring for 30 min. to decompose excess of dimethyl sulphate. After the addition of chloroform (350 ml.), the mixture was centrifuged, and the sediment washed with chloroform and rejected. The combined solutions consisted of an aqueous phase, a chloroform phase, and a gel which separated at the interface. The aqueous phase was extracted twice with chloroform and the extracts combined with the other chloroform solutions and the gel, and the suspension washed several times with water, whereupon further quantities of gel separated. Care was taken not to reject any of the gel with the water washings. The suspension of gel in chloroform was then washed with sodium ethylenediaminetetra-acetate (2% aqueous solution adjusted to pH 8; 3 × 1 l.), whereupon the gel disappeared. The resulting chloroform solution was washed with water (3 × 1 l.) and evaporated to dryness in a weighed flask (3·25 g.; Found: OCH_3, 31·70%). Fractional extraction of this product into light petroleum–chloroform mixtures of varying proportions (0—25% chloroform) gave a series of fractions which had varying methoxyl contents (OCH_3, 20·5—42·5%) but which were indistinguishable on hydrolysis and paper chromatography, and on methanolysis and gas–liquid or thin-layer chromatography. It was therefore concluded that the low methoxyl value found for the methylated desulphated polysaccharide was due to contamination.

Hydrolysis of Methylated Desulphated λ-Carrageenan and Separation of the Products.—The methylated polysaccharide (2·0 g.) was left overnight at room temperature with formic acid (70 ml.), whereupon the greater part of it dissolved. After the addition of water (70 ml.), the solution was heated in a closed flask on a boiling-water bath for 6 hr. Some residue remained undissolved. The suspension was concentrated to dryness in a rotary film evaporator, and water distilled repeatedly from the residue (*in vacuo*) until the solution was neutral. The mixture of methylated sugars, in the minimum volume of water, was applied to a charcoal–Celite column (60 × 6 cm. diameter) in the usual manner, and the column was eluted with a linear gradient from 1 to 5% aqueous ethyl methyl ketone over 10 litres of eluent. Fractions (50 ml.) were collected and analysed for carbohydrate using the phenol–sulphuric acid reagents, and selected fractions were examined further by paper chromatography and gas–liquid chromatography on the polyphenyl ether column after conversion into the equilibrium mixture of methyl glycosides. The fractions were then combined as follows: Tubes 1—124 contained only minute traces of di-*O*-methylgalactoses. Tubes 125—145 contained pure 2,4,6-tri-*O*-methyl-D-galactose (0·36 g.) which was identified by conversion into the crystalline anilide,[15] which after recrystallisation from ethanol was a white crystalline solid having m. p. and mixed m. p. 166°. No

[15] G. O. Aspinall, E. L. Hirst, R. W. Moody, and E. G. V. Percival, *J.*, 1953, 1631; G. O. Aspinall, E. L. Hirst, and A. Nicholson, *J.*, 1959, 1697.

other methylated sugar was detected in this fraction by gas chromatography. Tubes 146—180 contained a mixture (0·36 g.) of 2,3,6-tri-*O*-methyl-D-galactose and 2,4,6-tri-*O*-methyl-D-galactose as shown by gas chromatography, but with no other sugar present. Tubes 181—200 contained pure 2,3,6-tri-*O*-methyl-D-galactose (0·35 g.) which was identified by conversion into the crystalline lactone,[16] which after two recrystallisations from ethanol had m. p. and mixed m. p. 99—100°. No other methylated sugar was detected in this fraction by gas chromatography.

After these fractions had been collected, the column was eluted with 10% aqueous ethyl methyl ketone to displace 2,3,4,6-tetra-*O*-methyl-D-galactose (0·23 g.) which was identified by conversion into the anilide,[17] which after recrystallisation from ethanol had m. p. 188°.

Methylation of λ-Carrageenan.—The polysaccharide (15 g.) was dissolved in water (500 ml.) and reduced overnight with potassium borohydride (1 g.). Dimethyl sulphate (100 ml.) and sodium hydroxide (30% w/v; 300 ml.) were then added slowly and simultaneously with vigorous stirring during 6 hr., and the solution stirred for a further 18 hr. Addition of dimethyl sulphate and sodium hydroxide was repeated in this manner a further five times, and the polysaccharide was then isolated by dialysis and freeze-drying. A small portion was hydrolysed (1·5N-sulphuric acid at 100° for 3 hr.), and the solution neutralised (calcium carbonate) and then examined by paper chromatography. Methylation was judged to be incomplete, because galactose was detected as well as larger amounts of its methyl ethers. The methylation procedure was therefore repeated, after which the hydrolysate was found to contain only traces of galactose. The methylated λ-carrageenan was therefore isolated by evaporation of the dialysed solution, followed by freeze-drying and further drying at 60° *in vacuo* over phosphoric oxide (Yield: 10·7 g. Found: OCH_3, 9·8%; SO_3, 21·1%; 3,6-anhydrogalactose, 5·3%). The infrared spectrum of the product showed sulphate ester peaks that were similar in shape and position to those given by λ-carrageenan.

Hydrolysis of Methylated λ-Carrageenan and Separation of the Products.—Methylated λ-carrageenan (5·4 g.) was hydrolysed by heating on a boiling-water bath with 1·5N-sulphuric acid (100 ml.) for 3 hr. The hydrolysate was neutralised with calcium carbonate, filtered, and concentrated in a rotary film evaporator to a brown syrup, which was applied to a cellulose column (55 × 4 cm. diameter). The column was eluted with butanol half-saturated with water, and fractions (25 ml.) collected automatically. On the basis of paper chromatography, the fractions were combined into ten larger fractions which were evaporated to dryness. The following products were subsequently identified: Tubes 1—7 contained traces of tetra-*O*-methylgalactose (paper chromatography), together with degradation products arising from the hydrolysis. Tubes 8—13 contained 2,4,6-tri-*O*-methyl-D-galactose only (0·204 g.), which was identified by gas chromatography and by conversion into the crystalline anilide,[15] which after two recrystallisations from ethyl acetate was a white crystalline compound having m. p. and mixed m. p. 166°. Tubes 14—19 contained no carbohydrate. Tubes 20—22 contained 2,6-di-*O*-methyl-D-galactose only (0·050 g. $[\alpha]_D$, +77° in water), which was identified by gas chromatography, paper chromatography, and paper electrophoresis (see also below). Tubes 23—27 contained a mixture of 2,6- and 4,6-di-*O*-methyl-D-galactose (0·204 g.), which was completely separated on a cellulose column (55 × 4 cm. diameter) by elution with ethyl acetate–acetic acid–formic acid–water (18 : 3 : 1 : 2). The 2,6-di-*O*-methylgalactose so obtained was identified by conversion to the crystalline anilide which had an *X*-ray powder diagram identical to that of authentic material.

Tubes 28—36 contained pure 4,6-di-*O*-methyl-D-galactose (0·334 g.) which crystallised and after several recrystallisations from ethanol had m. p. 146—147°, $[\alpha]_D$, +120° (5 min.) ⟶ +74° (*c*, 0·35 in water) (Found: C, 46·14%; H, 7·49%. Calc. for dimethylhexose: C, 46·15%; H, 7·70). A sample of authentic 4,6-dimethyl-D-galactose was kindly provided by Dr. D. J. Bell.[18] Although the m. p.s were rather different (Dr. Bell's sample had m. p. 131—133°), the *X*-ray powder diagrams for the two substances were identical, the mixed m. p. was 142°, and the two equilibrium mixtures of methylglycosides gave on gas chromatography the same pattern of two peaks having *T* (see ref. 10) 2·89 (strong) and 4·2 (weak to medium) on the polyphenyl ether column. Demethylation of the dimethyl-sugar from carrageenan with boron trichloride,[19]

[16] W. N. Haworth, H. Raistrick, and M. Stacey, *Biochem. J.*, 1937, **31**, 640.
[17] P. Andrews, L. Hough, and J. K. N. Jones, *J.*, 1954, 806.
[18] J. S. D. Bacon, D. J. Bell, and J. Lorber, *J.*, 1940, 1147.
[19] T. G. Bonner, E. J. Bourne, and S. McNally, *J.*, 1960, 2929.

followed by paper chromatography, showed the presence of galactose only. The 4,6-di-*O*-methyl-D-galactose from carrageenan gave an osazone very readily on treatment with phenylhydrazine in the presence of sodium metabisulphite, m. p. 155°. Literature values for 4,6-di-*O*-methylgalactosazone ranging from m. p. 153 to 160—162° are listed by Smith and Montgomery.[20] Tubes 37—50 contained no carbohydrate. Tubes 51—54 contained 6-*O*-methylgalactose (0·055 g.) as shown by paper chromatography and paper electrophoresis, with traces of 2-*O*-methylgalactose. Tubes 55—59 contained a mixture of 2-*O*-methylgalactose and 3-*O*-methylgalactose (0·180 g.), as shown by paper chromatography and paper electrophoresis. Tubes 60—74 contained pure 3-*O*-methyl-D-galactose [21] (0·431 g.) which, crystallised and recrystallised from ethanol, had m. p. and mixed m. p. 143—144°.

The structural significance of the 2- and 6-*O*-methyl ethers of galactose is not clear, although it seems most likely that they arose through incomplete methylation of some of the 1,3-linked galactose units. In calculating the relative molar proportions of the various methylated sugars (these figures are quoted in the Introduction), the composition of the mixed fractions was estimated visually by inspection of paper chromatograms and the yields of the pure fractions corrected accordingly.

Examination of Alkali-modified λ-Carrageenan by Methylation and Hydrolysis.—Alkali-modified λ-carrageenan [1] was methylated with dimethyl sulphate and sodium hydroxide as described above, and the product (3·9 g. from 5·0 g. λ-carrageenan) was hydrolysed as before with sulphuric acid. The monomethylgalactose fraction of the hydrolysate was isolated by thick-paper chromatography, and examined further by electrophoresis; 3-*O*-methylgalactose was absent.

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[20] F. Smith and R. Montgomery, "The Chemistry of the Plant Gums and Mucilages," Reinhold Publ. Corp., New York, 1959.
[21] F. Reber and T. Reichstein, *Helv. Chim. Acta*, 1945, **28**, 1164.