340. *The Carrageenan System of Polysaccharides. Part I. The Relation between the κ- and λ-Components.**

By D. A. Rees.

Evidence is given that κ- and λ-carrageenan are structurally related. Two major differences between them are (i) that λ-carrageenan contains 1,4-linked D-galactose 2,6-disulphate units in place of the 3,6-anhydro-D-galactose which occurs in κ-carrageenan, and (ii) that κ-carrageenan contains a higher proportion of 4-sulphated D-galactose units. The possibility of a biological relation between the two polysaccharides is discussed.

Carrageenan, the family of sulphated galactans extracted with water from the red seaweeds *Chondrus crispus* and *Gigartina stellata*, has received much attention during the past twenty years. The first structural studies,[1,2] by methylation in conjunction with desulphation, showed that galactose units were present esterified with sulphuric acid at position 4 and combined in glycosidic linkage through position 3. Some branching through position 6 was also shown to occur. The early workers in Edinburgh and in Galway suspected that another sugar component was present as well as galactose, but this was not identified until later when derivatives of 3,6-anhydro-D-galactose were isolated by mercaptolysis.[3b] It was also shown at this time that carrageenan was a mixture of several polysaccharides.[4] The so-called κ-component was separated by utilising its tendency to gel in the presence of potassium ions, and it was assigned a provisional structure [3] based on an alternating chain of 1,3-linked β-D-galactosyl and 1,4-linked 3,6-anhydro-α-D-galactosyl units. The residual material ("λ-carrageenan") was further separated by fractional precipitation from aqueous ethanol into a main component and a polysaccharide containing L-galactose units.[5] There was also evidence for the presence of small amounts of xylan and floridean starch. Acetolysis of the λ-component showed that a high proportion of the galactose units were joined by α-1,3-linkages.[6] The sulphuric acid in κ- and in λ-carrageenan is believed to be esterified at least partly at position 4 of

* A brief account of part of this work has been published in *Chem. and Ind.*, 1961, 793.

[1] Buchanan, Percival, and Percival, *J.*, 1943, 51.
[2] Dewar and Percival, *J.*, 1947, 1622; Johnston and Percival, *J.*, 1950, 1994; Dillon and O'Colla, *Proc. Roy. Irish Acad.*, 1951, **54**, *B*, 51.
[3] (*a*) Percival, *Chem. and Ind.*, 1954, 1487; (*b*) O'Neill, *J. Amer. Chem. Soc.*, 1955, **77**, 6324.
[4] Smith, Cook, and Neal, *Arch. Biochem. Biophys.*, 1954, **53**, 192.
[5] Smith, O'Neill, and Perlin, *Canad. J. Chem.*, 1959, **33**, 1353.
[6] Morgan and O'Neill, *Canad. J. Chem.*, 1959, **37**, 1201.

galactose units because of the evidence obtained by Percival and his collaborators, and this view is consistent with interpretations of the infrared spectra.[7,8] More recently, supporting evidence for the presence of galactose 4-sulphate units has been obtained by partial hydrolysis (Dr. E. E. Percival, personal communication). The present paper describes a structural investigation of λ-carrageenan, with particular reference to the location of some of the sulphate groups. The polysaccharide sample, which was kindly given by Mr. L. Stoloff, was isolated from commercial *Chondrus crispus*.

" *Precursor Units* " *in* λ-*Carrageenan*.—Some other algal polysaccharides have been shown to contain sulphate ester groups so placed that S_N2 elimination within constituent units occurs under alkaline conditions. This reaction can be used to help in the identification of the sulphate esters, especially if potassium borohydride is added to minimise degradation of the polysaccharide chain.[9] When this method was applied to λ-carrageenan, about one-third of the sulphate ester was released as sulphate ion, with concomitant formation of 3,6-anhydro-D-galactose units; the latter were identified by isolation of the crystalline diethyl dithioacetal after complete hydrolysis of the polymer. It is suggested that the units which give rise to 3,6-anhydrogalactose (hereafter called " precursor units " or " precursor sulphate ") and constitute about 40% of the total structural units are mainly 1,4-linked D-galactose 2,6-disulphate, for the following reasons.

(i) Partial mercaptolysis of the alkali-treated λ-carrageenan gave carrabiose diethyl dithioacetal (4-*O*-β-D-galactopyranosyl-3,6-anhydro-D-galactose diethyl dithioacetal) which was identified by comparison of the crystalline hexa-*O*-acetate with authentic material. At least some of the sugar units under discussion are therefore joined glycosidically through position 4.

(ii) Partial acidic hydrolysis of λ-carrageenan and separation of the products led to the characterisation of D-galactose 6-sulphate. This substance was present in much smaller amount in the hydrolysate of alkali-treated λ-carrageenan, and it is therefore likely that 6-sulphate was eliminated from the polysaccharide during the treatment with alkali.

(iii) The rate of disappearance of precursor units when λ-carrageenan was heated in dilute hydrochloric acid was followed. Such disappearance occurs simultaneously in two ways under these conditions: by sulphate ester hydrolysis, and by glycosidic hydrolysis (illustrated for a galactose 6-sulphate unit: II ⟶ III and II ⟶ VI). Experiments with porphyran, which is known to contain galactose 6-sulphate units,[9] showed that these two factors contributed more or less equally to the disappearance (Fig. 1: Curves A and B).

It is theoretically possible that a polymer containing galactose 3-sulphate units would give 3,6-anhydrogalactose on treatment with alkali, but the rate of disappearance would be faster than in the case of the 6-sulphate, because both sulphate ester hydrolysis [10,11] and glycosidic hydrolysis [12] would be more rapid. It was found that the precursor units of carrageenan disappeared at a similar rate to those of porphyran (Fig. 1; curve B), suggesting that they are the same ester, *i.e.*, 6-sulphate. The curve for total sulphate release from λ-carrageenan (Fig. 1; curve C) shows an initial rapid hydrolysis of about one-third of the total sulphate. Evidence that this is due to hydrolysis of ester linkages in galactose 2-sulphate units will be discussed further in a later publication.[11]

(iv) λ-Carrageenan was largely resistant to periodate oxidation, in agreement with previous results.[5,6] When the periodate-oxidised polysaccharide was treated with alkali under the usual conditions, large amounts of 3,6-anhydrogalactose were produced (93% of the amount given by the non-oxidised polymer), suggesting that the majority of the precursor units had contained no glycol group oxidisable by periodate. This is consistent

[7] Bayley, *Biochim. Biophys. Acta*, 1955, **17**, 194.
[8] (*a*) Lloyd, Dodgson, Price, and Rose, *Biochim. Biophys. Acta*, 1961, **46**, 108; (*b*) Lloyd and Dodgson, *ibid.*, p. 118.
[9] Rees, *J.*, 1961, 5168.
[10] Barry and McCormick, *J.*, 1957, 2777.
[11] Rees, paper in preparation.
[12] Turvey and Clancy, *J.*, 1961, 2935.

with their being 1,4-linked galactose 2,6-disulphate (I). When the polysaccharide was treated with hot dilute acid for a short time, to effect removal of the acid-labile ester mentioned in the previous paragraph, it gave a mixture of oligosaccharides in which the precursor grouping was still present in large amounts (about 45% of the original quantity), but apparently in modified form since the greater part of it (73% of that remaining) could now be oxidised by periodate, *i.e.*, contained a glycol group. These observations are explained if the precursor is 1,4-linked galactose 2,6-disulphate. Thus, during acidic treatment there is a rapid loss of 2-sulphate (I ⟶ II) with smaller amounts of 6-sulphate

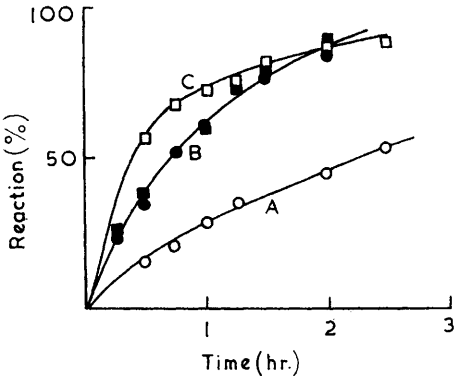


FIG. 1. Comparison of total sulphate release from (A) porphyran and (C) λ-carrageenan; and precursor sulphate disappearance from porphyran (B, ●) and λ-carrageenan (B, ■). For conditions see text.

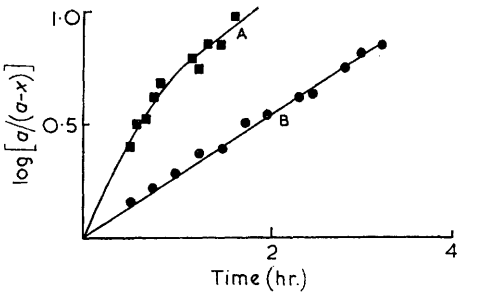
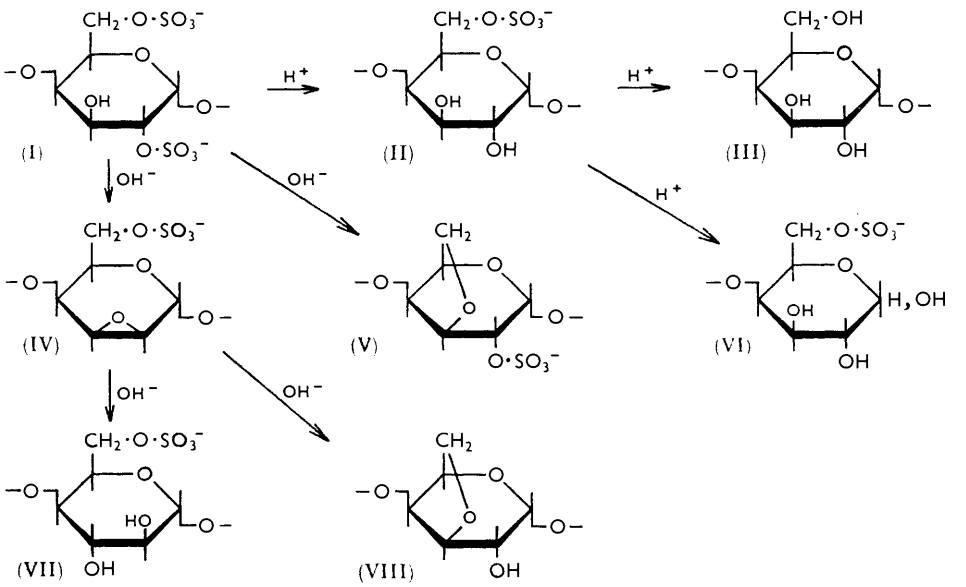


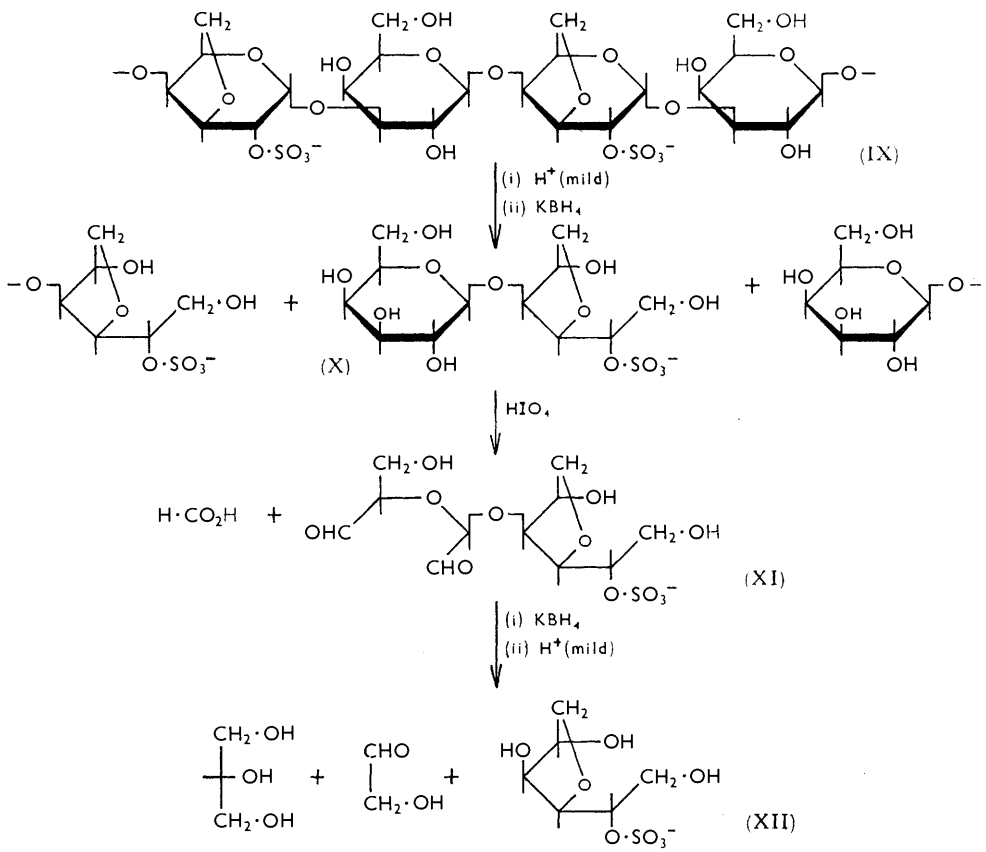
FIG. 2. Comparison of the sulphate-release curves for (A) alkali-treated λ-carrageenan and (B) a typical natural κ-fraction (from *F. fastigiata*). For conditions see text.

hydrolysis (II ⟶ III) and glycosidic hydrolysis (II ⟶ VI). The removal of the 2-sulphate exposes the 2,3-diol, which can therefore be oxidised by periodate. A proportion of free glycol groups probably also arise by glycosidic hydrolysis, thus exposing



the 3,4-diol. The important point is that it is impossible to explain these observations on the assumption that the precursor unit is a 3-monosulphate.

(v) The scheme proposed for the alkali-catalysed reaction requires that 3,6-anhydrogalactose 2-sulphate (V) be present in the product. The first indication that this might be so was when the 3,6-anhydrogalactosidic linkages, although very labile to acid, were found to be appreciably less labile than the corresponding linkages in polysaccharides in which the anhydrosugar presumably carries no 2-substituent. Thus, the 3,6-anhydrogalactosidic linkages in porphyran and a κ-fraction of *Furcellaria fastigiata* were hydrolysed rapidly and at the same rate in 0·25N-hydrochloric acid at 60° (half-life, 0·69 hr.), whereas the hydrolysis of the carrageenan derivative was slower (half-life, 3·00 hr.). Turvey and Clancy [12] have previously found that sulphate ester groups, like toluene-*p*-sulphonyl ester groups,[13] stabilise the glycosidic linkage of the sugar unit to which they are attached. One interpretation of the observed stabilisation (though there are several others possible) is, therefore, that the anhydro-sugar units are sulphated at position 2 (sulphation is unlikely at position 4 because the evidence given above suggests that this is engaged in glycosidic linkage). In order to isolate a sulphated derivative of 3,6-anhydrogalactose and thus to obtain more direct evidence for the presence of the 2-sulphate, it was necessary to depolymerise the polysaccharide. Since partial acid fragmentation as normally used could not be applied because of the lability of the 2-ester linkage, the illustrated sequence of degradations (IX ⟶ XII) was followed.



The assumption was made in devising this sequence, that some of the 3,6-anhydrogalactose 2-sulphate units were separated in the chain by one galactose unit (IX).

[13] Dr. A. Rudowski, personal communication; Wood and Fletcher, *J. Amer. Chem. Soc.*, 1958, **80**, 5242.

Despite the stabilisation of the 3,6-anhydrogalactosidic linkages by the 2-sulphate, it was possible to effect their hydrolysis by using very mild conditions,[14] and after reduction of the newly exposed reducing groups the hydrolysate was subjected to the degradation procedure of Smith and his co-workers [15] (X ⟶ XII). A small portion of the mixture of products was examined after complete acidic hydrolysis; large quantities of 3,6-anhydrogalactitol were then found, having survived the periodate treatment. This shows that the anhydro-sugar units in the polysaccharide must have carried a 2-substituent, in addition to the glycosidic 4-substituent. The mixture of products yielded (after a purification procedure which involved considerable losses) a substance with the expected properties of 3,6-anhydrogalactitol 2-sulphate. Although this structure is provisional until the compound has been compared with material synthesised by a definitive route, all the available observations are consistent with the expected structure. The compound was obtained as a chromatographically and electrophoretically homogeneous syrup. Complete acidic hydrolysis gave equimolar quantities of free sulphate and 3,6-anhydrogalactitol. The latter was determined by periodate oxidation and identified by (i) its paper chromatographic mobility, (ii) limited periodate oxidation to 2,5-anhydrolyxose which was identified by its characteristic behaviour on paper chromatograms, and (iii) oxidation of the hydrolysate in dilute aqueous sodium periodate at room temperature when there was rapid oxidation in which 2 mol. of oxidant were consumed and 1 mol. of formaldehyde was liberated, followed by slow over-oxidation. This evidence suggests that the compound was a sulphate ester of 3,6-anhydrogalactitol, and from the further fact that it consumed one mol. of periodate without producing formaldehyde, it appears to be 3,6-anhydrogalactitol 2-sulphate (the 1-sulphate not being considered as a possible structure).

(vi) The infrared absorption spectra of carbohydrate sulphate esters show a general absorption band at 1240—1250 cm.$^{-1}$, and in addition, bands that are thought to be specific for the type of sulphate ester present [8] at 820 cm.$^{-1}$ (sulphated primary hydroxyl group), 830 cm.$^{-1}$ (sulphated equatorial secondary hydroxyl group), and 850 cm.$^{-1}$ (sulphated axial secondary hydroxyl group). The spectrum of λ-carrageenan showed a very broad band at 810—860 with a maximum at *ca.* 827 cm.$^{-1}$, suggestive of a mixture of all three types of sulphate ester. This is consistent with the presence of galactose 2,6-disulphate (the 2-sulphate being equatorially disposed in the stable C1 conformation and the 6-sulphate attached to a primary alcohol), together with galactose 4-sulphate (the esterified hydroxyl group here being axial in the stable C1 conformation). In contrast, the alkali-modified derivative showed a fairly sharp band at *ca.* 850 cm.$^{-1}$, indicating that all the sulphate remaining is here attached to axial secondary hydroxyl groups. This too is expected, because the primary hydroxyl group was de-esterified in alkaline treatment and the 2-sulphate is now axial, being substituted in a unit of 3,6-anhydrogalactose which is probably in the 1C conformation in polysaccharides of this type.[7]

(vii) A 2,6-disulphated galactose unit within the polysaccharide (I) can (in theory) react in the presence of alkali in several ways. It is not to be expected that the reaction should proceed exclusively by any one path, but the results of Rao and Smith,[16] who studied the action of alkali on methyl α-D-galactopyranoside 2,6-ditoluene-*p*-sulphonate, suggest that the most important reaction would be direct conversion into 3,6-anhydrogalactose 2-sulphate (V) rather than epoxide formation (IV) followed by ring opening, to give either non-sulphated 3,6-anhydrogalactose (VIII) or D-idose 6-sulphate (VII). If side reactions involving the epoxide intermediate occur with carrageenan, they would result in the liberation of more than one mole of sulphate for every mole of 3,6-anhydride formed. In fact it has been repeatedly observed that there is not an exact correspondence between the molar amounts of the two products, but an excess of free sulphate amounting to about

[14] D. A. Rees, Ph.D. Thesis, University of Wales, 1959.
[15] Goldstein, Hay, Lewis, and Smith, Amer. Chem. Soc. Meeting, Boston, April 1959, Abs. Papers 3D.
[16] Rao and Smith, *J.*, 1944, 229.

15% of the total released, *i.e.*, about 5% of the sulphate ester initially present. This figure represents an upper limit rather than the exact extent of such side reactions, since there are alternative possible explanations for the disparity, such as the presence of other alkali-labile sulphate groups in the polysaccharide, or the occurrence of a certain amount of O–S cleavage of the 4- and 2-sulphate groups under the alkaline conditions. A further important conclusion is that the stoicheiometry of the reaction is consistent with the presence of galactose 2,6-disulphate (I) rather than galactose 3,6-disulphate or 2,3,6-trisulphate. The last two sulphates, though theoretical possibilities, would each give 3,6-anhydrogalactose at the expense of at least 2 mol. of esterified sulphate.

The Relation between the κ- and λ-Components.—Polysaccharides of the κ-carrageenan type have been recognised in a large number of red algae.[3b,17,18] The characteristic properties of this group are (i) a general resemblance in the proportions of galactose, 3,6-anhydrogalactose, and ester sulphate present, (ii) partial fragmentation to carrabiose derivatives, (iii) gelation or flocculation on addition of potassium salts, and (iv) hydrolysis by an enzyme (κ-carrageenase) thought to be specific for this type of polysaccharide. Alkali-modified λ-carrageenan resembles these polysaccharides in giving carrabiose derivatives on fragmentation, and in that its analytical figures conform to the general pattern (Table 1). In addition, a substantial gelatinous precipitate was observed when

TABLE 1.

Comparison of alkali-modified λ-carrageenan with some naturally occurring κ-fractions.

Source.	Molar ratio (galactose : 3,6-anhydrogalactose : total sulphate)
Hypnea speciferea [18b]	1 : 0·8 : 0·7
Furcellaria fastigiata [17]	1 : 0·8 : 0·8
Chondrus crispus (a) [4]	1 : 0·7 : 1·0
Chondrus crispus (b) [4]	1 : 0·9 : 1·0
Chondrus crispus (c) [3]	1 : 0·8 : 1·2
Alkali-modified λ-carrageenan (*Chondrus crispus*)	1 : 0·6 : 1·2

potassium chloride was added at a concentration of 0·25M to a solution of the alkali-modified λ-carrageenan, little further material being deposited when the concentration was increased to M. 0·3M-Sodium chloride gave only a small precipitate, indicating that little of the precipitate with potassium ions was formed by non-specific coagulation of colloidal material such as could have been induced by any electrolyte.

An important observation in relation to the analytical figures in Table 1 is that, although all the samples listed are sulphated to roughly the same extent, the distribution of ester groups between different sites on the polysaccharide molecule might differ from sample to sample. This was first suspected because the acid-lability of the 3,6-anhydrogalactosidic linkages in a κ-fraction of *Furcellaria fastigiata* suggested the absence of the stabilising 2-sulphate that is postulated in alkali-modified λ-carrageenan. It appears[11] that the curve showing the liberation of free sulphate when a carbohydrate sulphate is heated with acid can serve as a " fingerprint " to help in the characterisation of the ester linkages present. The product from the alkaline treatment of λ-carrageenan was therefore compared by this means with some naturally occurring κ-fractions, isolated from *Chondrus crispus*, *Furcellaria fastigiata*, and *Hypnea musciformis*. These three samples were kindly provided by Dr. W. Yaphe, and are described in an earlier paper.[9] The results are given in Fig. 2. Although the three naturally occurring κ-fractions are very similar in the behaviour of their sulphate esters towards hydrolysis, there is a sharp contrast between this group and the alkali-modified λ-carrageenan. The curves can be tentatively interpreted as follows. The alkali-modified λ-carrageenan contains at least two esters since there are two phases in the hydrolysis, whereas there is no evidence for

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

[18] (*a*) Araki and Hirase, *Bull. Chem. Soc. Japan*, 1956, **29**, 770; (*b*) Clingman and Nunn, *J.*, 1959, 493; (*c*) Yaphe, *Canad. J. Bot.*, 1959, **37**, 751.

the presence of more than one in any of the other three preparations. A relatively acid-stable ester is present in all polysaccharides and, from previous evidence, this is probably galactose 4-sulphate. From the intercept on the graph it is deduced that this sulphate represents only about 40% of the total ester present in alkali-modified λ-carrageenan, the other 60% being an acid-labile sulphate. Since 3,6-anhydrogalactose 2-sulphate is postulated as present in alkali-modified λ-carrageenan in substantial amounts, this is probably the acid-labile ester revealed in this polysaccharide but not in any of the natural κ-fractions. At first sight this deduction might seem inconsistent with the provisional generalisation [11] that the esters derived from axial secondary hydroxyl groups are relatively acid-stable. As noted in the above discussion of the infrared spectra, any 2-sulphate group attached to the 3,6-anhydrogalactose units in the polysaccharides would probably be axial. However, the 3,6-anhydrogalactosidic linkages in the polysaccharide are so acid-labile that they are probably quickly hydrolysed at the beginning of the determination of the acid-release curve. Since no firm prediction can be made regarding the favoured conformation of the 3,6-anhydrogalactose units in such a partially hydrolysed polysaccharide, it is not inconsistent with the generalisation [11] to attribute the rapid reaction to the 2-ester. It is, therefore, concluded that the unmistakable difference between the sulphate release curves can be traced to the occurrence in large amounts of 3,6-anhydrogalactose 2-sulphate units in the alkali-modified λ-carrageenan, and their virtual absence from the naturally occurring κ-fractions.

In summary, it seems that a polysaccharide that is related to κ-carrageenan can be prepared from λ-carrageenan by controlled treatment with alkali. It is therefore conceivable that the main component of λ-carrageenan is a biological precursor of the κ-polysaccharide, normally differing from it in containing (i) galactose 2,6-disulphate units in place of 3,6-anhydrogalactose and (ii) a lower proportion of 4-sulphated galactose units. This suggests the presence in the living seaweed of several enzymes that metabolise sulphate esters, including one that effects the 6-sulphate ⟶ 3,6-anhydride conversion *in vivo* in a manner analogous to that of alkali in these experiments. Such an enzyme is already known to catalyse this transformation in the L-series of sugars.[19] Some work by previous authors needs however to be reconciled with this view. Bayley's [7] sample of λ-carrgeenan seems to resemble that used here because the published infrared spectrum shows a rather broad peak in the 810—860 cm.$^{-1}$ region; and the whole carrageenan of Buchanan, Percival, and Percival [1] contained some alkali-labile sulphate, which might well have been " precursor sulphate." On the other hand, investigators using the methylation technique have failed to find evidence for 1,4-linkages in whole carrageenan [1,2] and Lloyd *et al.*[8a] describe the spectra of a number of λ-fractions as having sharp bands at 850 cm.$^{-1}$ with no trace of absorption at 820 cm.$^{-1}$. The most likely explanation of these differences seemed that there is variation in the composition of λ-carrageenan according to the source from which it is extracted, and a brief survey was therefore undertaken. Three samples of *Gigartina stellata* were obtained (through the courtesy of Dr. Elsie Conway and Miss Kathleen Atkinson) from Millport, Isle of Cumbrae, Scotland, at different seasons. Fractionation of the derived whole carrageenan by the usual method [4] gave λ-fractions which are compared in Table 2 with samples (kindly supplied by Mr. L. Stoloff) prepared from American commercial *Chondrus crispus*. The yields of the λ-components from *Gigartina* are low, despite the fact that the total recovery was about the usual (85%) in all three cases. Workers with *Chondrus* of American origin normally expect a yield of λ-carrageenan of about 50%. The three British λ-components, unlike the American, contained appreciable quantities of combined 3,6-anhydrogalactose, with correspondingly lower amounts of precursor (Table 2). It seems that there might be here a relation similar to that found for *Porphyra* species [20] between the amount of 3,6-anhydrogalactose and that of its biological precursor, but insufficient samples of *Chondrus* and *Gigartina* have

[19] Rees, *Biochem. J.*, 1961, **81**, 347.
[20] Rees and Conway, *Biochem. J.*, 1962, **84**, 411.

TABLE 2.

Comparison of some λ-components.

Source	Yield of λ-component	Molar ratio (non-precursor sulphate : precursor : 3,6-anhydride)
Chondrus crispus	—	1 : 0·56 : 0·05
Chondrus crispus	—	1 : 0·49 : 0·05
G. stellata (May)	33	1 : 0·20 : 0·39
G. stellata (August)	20	1 : 0·22 : 0·39
G. stellata (November)	13	1 : 0·20 : 0·34

yet been examined to inspire confidence. However, it can be concluded that for certain carrageenans the sharp κ/λ-fractionation is not obtained, but, instead, a λ-fraction is isolated in diminished yield and with properties intermediate between those of the " normal " κ- and λ-polysaccharide. These " λ-fractions " gave fairly sharp bands in their infrared spectra at 850 cm.$^{-1}$, presumably because the proportion of other sulphate ester was not sufficient grossly to distort the shape of the axial sulphate peak. It is concluded that the " λ-fractions " of Lloyd *et al.*[8] could have been of this type. Moreover, it is now seen to be possible that the early workers used whole carrageenan samples in which the amount of 1,4-linked galactose 2,6-disulphate units was sufficiently small to escape detection by their methods.

The results of Morgan and O'Neill [6] are not yet reconciled with the present conclusions. By a study of acetolysis fragments they obtained evidence for a high proportion of α-1,3-linkages in λ-carrageenan, but recognised no fragments containing 1,4-linkages. Since the 3,6-anhydrogalactose content of their polysaccharide was low (1—2%), it was presumably of the " true λ " type. The reason for their failure to obtain fragments containing 1,4-linkages is being investigated further.

EXPERIMENTAL

General and Analytical Methods.—Most of these have been described previously.[9,20] Analysis for galactose was carried out directly with the phenol–sulphuric acid reagents.[21] Reduction of periodate was measured by Aspinall and Ferrier's spectrophotometric method.[22] Formaldehyde was estimated colorimetrically with chromotropic acid.[23] Charcoal used for chromatography, unless stated otherwise, was May and Baker's " Decolourising " grade; columns were prepared without the addition of " Celite." Concentration of solutions was carried out under diminished pressure at 40° in a rotary film evaporator. Whatman 3 MM paper was used for preparative chromatography and electrophoresis. The buffer used for paper electrophoresis was prepared by adjusting 0·05N-acetic acid to pH 7·5 by the addition of pyridine.

λ-*Carrageenan.*—The material used for most of this work was prepared from commercial *Chondrus crispus* and kindly given by Mr. L. Stoloff, of Marine Colloids Inc., Rockland, Maine, U.S.A. It had 44·5% of galactose, 25·6% of sulphate (as SO_3) and 1·6% of 3,6-anhydrogalactose. $[\alpha]_D$ was 81° ± 5° (*c* 0·2 in water). Complete acidic hydrolysis followed by paper chromatography (spray: *p*-anisidine hydrochloride) showed the presence of galactose only, with traces of xylose and fucose.

Alkaline Treatment of λ-*Carrageenan.*—The following is a typical experiment. Polysaccharide that had been pre-dried at 60° *in vacuo* over phosphorus pentoxide for 24 hr. (2·82 g.) was dissolved in water (250 ml.), and potassium borohydride (0·5 g.) was added. After 20 hr. at room temperature, 3N-sodium hydroxide (125 ml.) was added, with a further quantity of potassium borohydride (1·5 g.), and the mixture was heated at 80° for 7 hr. Analysis showed that 3,6-anhydrogalactose had been formed equivalent to 14·8% (anhydro-basis) of the weight of the original sample. The solution was transferred to a dialysis sac in a beaker of water (1 l.), and after 24 hr. no 3,6-anhydrogalactose could be detected in the diffusate (limit of detection of the test about 1% of the total present). The solution was dialysed exhaustively against running tap water, concentrated, and freeze-dried (2·81 g., 14·2% of 3,6-anhydride and 15·9%

[21] Dubois, Gilles, Hamilton, Rebers, and Smith, *Analyt. Chem.*, 1956, **28**, 350.
[22] Aspinall and Ferrier, *Chem. and Ind.*, 1957, 1216.
[23] Frisell, Meech, and Mackenzie, *J. Biol. Chem.*, 1954, **207**, 709.

of sulphate). It is calculated that 82% of the sulphate loss resulted in the formation of 3,6-anhydrogalactose.

Complete Mercaptolysis.—The procedure is based on that of Painter.[17] Alkali-modified λ-carrageenan (2·7 g., containing 14·1% of 3,6-anhydride) was stirred at 0° with ice-cold concentrated hydrochloric acid (30 ml.). When the mixture became a slurry, ethanethiol (25 ml.) was added, the flask was closed, and the contents were allowed to come to room temperature and stirred for 96 hr. The solution was poured into chilled methanol (3 vol.), neutralised with lead carbonate, filtered, concentrated to 100 ml., and extracted repeatedly with ether until only a weak Seliwanoff test was given by the ether phase (12 × 100 ml.). The combined ether extracts were concentrated, dried (Na_2SO_4), concentrated further to 50—100 ml., and left at 0° for 24 hr. Crystals were deposited (150 mg.), presumably galactose diethyl dithioacetal (m. p. after several recrystallisations, 141·5°), which were not examined further. The further material (two crops, combined weight 410 mg.) that crystallised on addition of light petroleum (b. p. 40—60°) was shown by paper chromatography to consist mainly of 3,6-anhydrogalactose diethyl dithioacetal. The yield of this material (58%) was about that expected, since Painter's yield at this stage was 62%. Recrystallisation from ether–light petroleum gave pure material, m. p. and mixed m. p. 111·5—112·5°, $[\alpha]_D$ −9·5° (*c* 1·8 in H_2O).

Partial Mercaptolysis.—Alkali-modified material (9·5 g.) was treated with concentrated hydrochloric acid (70 ml.) and ethanethiol (25 ml.) as previously, except that, after mixing, the reactants were stirred at 0° for 10 hr. and then at room temperature for 40 hr. The product was treated with methanol and lead carbonate, the solution filtered and taken to dryness, and the residue dissolved in water (80 ml.). After 24 hr. the solution was filtered and extracted continuously with ether for 3 days. The aqueous layer was put on a column of coconut charcoal[17] (29 × 6·5 cm. diam., 1 lb. dry wt.) which was washed with ethanol–water (1 : 4) (7 l.) and then with 50% aqueous ethanol (15 l.) which slowly eluted a Seliwanoff-positive material. This effluent was concentrated to a syrup (0·65 g.), shown by paper chromatography to be almost pure carrabiose diethyl dithioacetal. This accounts for 15% of the 3,6-anhydrogalactose originally present, which is rather low in comparison with the yields of other workers.[3b,17] It is thought that substantial losses were incurred through lack of experience with the particular type of charcoal; a much larger column than necessary was used, and the desired material was displaced only with difficulty, and possibly incompletely. Hexa-*O*-acetyl-4-*O*-β-D-galactopyranosyl-3,6-anhydro-D-galactose diethyl dithioacetal was prepared from the product by Painter's method,[17] and after repeated recrystallisation from aqueous ethanol had m. p. and mixed m. p. 119—120°, $[\alpha]_D$ −4° (*c* 1·3 in $CHCl_3$). The *X*-ray powder photograph was identical with that of an authentic specimen.

D-*Galactose* 6-*Sulphate in the Polysaccharides.*—λ-Carrageenan (3 g.) was heated with 0·3N-sulphuric acid (150 ml.) at 100° for 5 hr. After neutralisation with barium carbonate the solution was passed through Amberlite IR-120 resin (H^+ form) and then IR-400 (standard grade, acetate form), Acidic sugars were displaced from the latter column with 0·5N-sulphuric acid (about 1 l.). The effluent was neutralised with barium carbonate, concentrated, passed through a column of IR-120 resin (H^+ form), and evaporated with additions of water until the distillate was neutral. It was then neutralised by aqueous ammonia and put on a charcoal column (7 × 3 cm. diam.) which was washed with water. Galactose 6-sulphate was thus obtained contaminated with salts and a sulphated disaccharide. The former were removed by passing the solution down IR-120 resin (H^+ form), shaking the effluent with 5% tri-n-octylamine in chloroform, and finally washing it with chloroform. The product, which remained in the aqueous phase, was purified by thick-paper chromatography in ethyl acetate–acetic acid–formic acid–water (18 : 8 : 3 : 9) (16 hr.), giving an ammonium salt (93·5 mg.), $[\alpha]_D$ +51° (*c* 0·4 in H_2O). The degree of polymerisation[24] was 1·00, and the molar ratio galactose : sulphate was 1·02 : 1·00. When this material was left at room temperature in the dark in 0·01M-sodium metaperiodate, 3·5 mol. of oxidant were consumed after 24 hr., with the production of negligible amounts of formaldehyde. When the sugar sulphate was heated at 100° in 0·25N-hydrochloric acid, hydrolysis of the sulphate ester linkage obeyed first-order kinetics, the half-life being 1·61 hr. These properties characterise the product as D-galactose 6-sulphate.[11,25]

Similar quantities (about 30 mg.) of λ-carrageenan and alkali-modified λ-carrageenan were separately hydrolysed in 0·4N-sulphuric acid at 100° for 3 hr., neutralised with barium carbonate,

[24] Peat, Whelan, and Roberts, *J.*, 1956, 2258.
[25] Turvey and Rees, *Nature*, 1961, **189**, 831.

and passed through a small charcoal column (5 × 2 cm. diam.) which was washed with water. The effluent was concentrated and examined by paper electrophoresis. The spot corresponding to monosaccharide sulphates was rather more intense in the hydrolysate of the λ-carrageenan than in that of the alkali-modified derivative (compared at similar concentrations of neutral material). The monosaccharide sulphate fraction was separated by thick-paper electrophoresis and examined by paper chromatography.[26] That from λ-carrageenan contained chiefly galactose 6-sulphate, with small amounts of galactose (?) 4-sulphate, whereas the product from alkali-modified λ-carrageenan contained mainly the (?) 4-sulphate. It is concluded that the amount of 6-sulphate in the polysaccharide is greatly diminished by alkali treatment, although some remains. It is not clear whether this is due to incomplete alkaline elimination or to the presence of 6-sulphated units in some other linkage.

Acidic Hydrolysis of Sulphates.—(i) *Total sulphate release.* Samples of the polysaccharide in 0·25N-hydrochloric acid (0·250 ml.) were heated in sealed ampoules on a boiling-water bath. Ampoules were removed from time to time, and the contents were exactly neutralised by 2·50N-sodium hydroxide (0·025 ml.). A trace of Cetavlon was then added, followed by chloroaminodiphenyl reagent (0·250 ml.), and the sulphate present was determined spectrophotometrically by Jones and Letham's method.[27] It is not possible by this procedure to study the reaction for the first 30 minutes because oligosaccharide sulphates of high molecular weight are present which interfere with the determination. When polysaccharides containing $\geqslant 10\%$ of 3,6-anhydrogalactose were being studied, a correction was applied to allow for the ultraviolet absorption of its degradation products.

(ii) *Disappearance of precursor units.* The procedure was similar to that used for following total sulphate release, except that, after neutralisation, potassium borohydride (5 mg.) was added and the solution left overnight. 3N-Sodium hydroxide (0·5 vol.) was then added, together with more potassium borohydride (5 mg.), and the usual estimation of precursor units was carried out.[20] In the case of porphyran, calculation of the rate of disappearance of precursor was complicated because combined 3,6-anhydrogalactose was present initially in considerable amounts, and this disappeared progressively during the reaction owing to acid degradation. In order to avoid the need for a correction factor a degraded 3,6-anhydride-free preparation was used. This material was Peat and Rees's " oligosaccharide A." [28]

Comparison of the Rates of Hydrolysis of Some 3,6-*Anhydrogalactosidic Linkages.*—The polysaccharide in 0·25N-hydrochloric acid solution was left in a thermostat-bath at 60° and samples were withdrawn from time to time into test tubes containing an excess of calcium carbonate. An excess of potassium borohydride was added, and the tubes were left for 16 hr. at room temperature. Portions of each suspension were analysed for 3,6-anhydrogalactose, and the results calculated on the assumption that every 3,6-anhydrogalactosidic linkage that was hydrolysed gave rise (after reduction) to an anhydrogalactitol unit which was detected by a decrease in the amount of 3,6-anhydrogalactose present.

Isolation of 3,6-*Anhydrogalactitol* 2-*Sulphate.*—λ-Carrageenan (3·0 g.) was dissolved in water (350 ml.) and reduced with an excess of potassium borohydride at room temperature overnight. Solid sodium hydroxide (14 g.) was dissolved in the solution which was heated at 80° for 7 hr., cooled, and exhaustively dialysed against water. Sulphuric acid was added to 0·25N, and the mixture left in a thermostat-bath at 60° for 12 hr., neutralised with barium carbonate, and filtered. An excess of potassium borohydride was added and after 24 hr. at room temperature only a weak Seliwanoff test was given by a portion of the mixture, showing that virtually all the 3,6-anhydrogalactosidic units had been hydrolysed and were now converted into terminal 3,6-anhydrogalactitol units. The solution was treated with Amberlite IR-120 resin (H^+ form) to destroy the excess of borohydride and remove the cations, neutralised with aqueous ammonia and concentrated to 150 ml. Periodic acid (5 g.) was dissolved in the solution, which was then left at room temperature for 6 hr., after which an excess of oxidant still remained (benzidine test). After neutralisation with barium hydroxide the precipitate was removed on the centrifuge, an excess of potassium borohydride added, and the solution was left at room temperature for 12 hr. and then adjusted to 0·3N with respect to sulphuric acid. After a further 48 hr. at room temperature it was neutralised with barium carbonate, filtered, concentrated to small volume, and placed on a charcoal column (16 × 2·5 cm.) which was eluted with water. This

[26] Rees, *Nature*, 1960, **185**, 309.
[27] Jones and Letham, *Chem. and Ind.*, 1954, 662.
[28] Peat and Rees, *Biochem. J.*, 1961, **79**, 7.

fractionation technique was used to remove most of the salt and oligosaccharide material. A small portion of each column fraction was hydrolysed by 0·5N-sulphuric acid at 100° for 1 hr and then neutralised with Amberlite IRA-45 resin (OH^- form), and the product was submitted to the following rapid test for the detection of 3,6-anhydrogalactitol. Several small drops of the hydrolysate were superimposed in the usual manner on a strip of paper mounted in the electrophoresis apparatus. The strip was moistened with buffer in the usual way, only the paper in the immediate vicinity of the spot of test substance remaining dry. A small drop of chilled 0·1M-periodic acid was superimposed on the test substance and a potential gradient of 15 v/cm. immediately applied to the paper. After 15 min., the strip was dried, sprayed with *p*-anisidine hydrochloride reagent, and heated. The appearance of a bright, rather fluorescent red spot near the point of application was a positive result. The column fractions which were thus shown to contain combined 3,6-anhydrogalactitol were united and concentrated to small volume, and the acidic material was separated by preparative paper electrophoresis, the solution being neutralised with aqueous ammonia before evaporation to dryness (280 mg.). Chromatography of the product with ethyl acetate–acetic acid–formic acid–water (18 : 3 : 1 : 4) as solvent and periodate–benzidine as spray showed two components, having R_F values relative to 3,6-anhydrogalactitol of 0·15 and 0·32, respectively. The faster-moving material was isolated by preparative paper chromatography in the same solvent and obtained as a syrup (72 mg.) that still contained some inorganic salts but no free sulphate and had $[\alpha]_D$ +10° (*c* 1·1 in H_2O). Only a single carbohydrate component appeared to be present because the substance gave a single compact spot on paper chromatography and paper electrophoresis (mobility relative to glucose 3-sulphate, 1·30) and showed only 3,6-anhydrogalactitol on hydrolysis and paper chromatography. The presence of 3,6-anhydrogalactitol in the hydrolysate was confirmed by limited periodate oxidation on a paper electrophoretogram as described above, but after drying the paper, instead of spraying, the material on the starting line was eluted and chromatographed in butanol–ethanol–water (5 : 1 : 4). Spraying the chromatogram with *p*-anisidine hydrochloride and heating disclosed a fast-moving, streaky, bright red spot (believed to be due to 2,5-anhydrolyxose), similar to that obtained from authentic 3,6-anhydrogalactitol. A part of the product (1·07 μmoles, measured by the sulphate ester content) was heated for 12 hr. on a boiling-water bath in 0·20N-hydrochloric acid (0·125 ml.), in a sealed tube. After neutralisation by 2·50N-sodium hydroxide (0·010 ml.), 0·05M-sodium periodate was added (0·10 ml.), and the mixture set aside in the dark at room temperature for several hours.

The following values were observed:

Time (hr.)	3	6	24	36
IO_4^- reduced (mol.)	1·7	2·2	3·2	3·5
CH_2O released (mol.)	0·90	0·95	1·14	1·25

Another part of the product (1·25 μmoles), dissolved in 0·010M-sodium periodate (1·0 ml.), was found to reduce 1·0 mol. of periodate in 12 hr., producing negligible amounts of formaldehyde. No further increase in periodate consumption was observed in the following 12 hr.

Periodate Oxidations.—A 0·3% solution of λ-carrageenan in 0·05M-sodium periodate was left at room temperature for 72 hr., after which the consumption of oxidant corresponded to <1 mole per 10 galactose units in the polymer. Ethylene glycol was added to destroy the excess of periodate, and the solution was analysed as usual for precursor units; it was found that only 7% of those initially present had been oxidised. In a separate experiment, a 0·6% λ-carrageenan solution (5 ml.) was adjusted to 0·25N with respect to hydrochloric acid and heated at 100° for 30 min. After neutralisation by sodium hydroxide the solution was diluted to 10 ml. and analysed for precursor units. 0·1M-Sodium periodate (1 vol.) was added, and after 72 hr., when the periodate consumption corresponded to about 1·3 mol. per galactose unit, the reaction was stopped by ethylene glycol, and the product was analysed for precursor units. The results are given in the Discussion.

Extraction of Some Samples of Gigartina stellata.—Each seaweed sample was extracted repeatedly with boiling water, and the combined solutions were exhaustively dialysed against running tap water, concentrated, and freeze-dried. The product was further dried *in vacuo* over phosphorus pentoxide at 60° for 24 hr., and a sample (2·0 g.) taken and dissolved in water (1 l.). Solid potassium chloride (20 g.) was added to the solution with stirring, and after 1 hr. the gelatinous precipitate was removed on the centrifuge. The precipitate and the supernatant solution were each worked up in the usual way,[4] except that the polysaccharides were finally

isolated by freeze-drying rather than by precipitation from aqueous ethanol. Each product was dried *in vacuo* at 60° over phosphorus pentoxide, weighed, and analysed for 3,6-anhydrogalactose, total sulphate, and precursor units. The results are given in the Discussion.

The effect of higher concentrations of potassium chloride on solutions of the various λ-fractions was briefly examined as follows. The polysaccharide solution (0·1—0·2%; 10 ml.) was mixed with solid potassium chloride (1 g.), and after 1 hr. at room temperature the precipitate was removed on the centrifuge. The 3,6-anhydrogalactose content of the supernatant solution was then compared with that of the solution before addition of potassium chloride. The first λ-component (from seaweed collected in May) gave only a slight precipitate and there was no significant decrease in the amount of 3,6-anhydride in solution. The second (August) gave a slight precipitate which (by difference) contained 25% of the anhydrosugar present. The third (November) gave a considerable precipitate which contained 65% of the anhydrosugar.

I thank Professor E. L. Hirst, C.B.E., F.R.S., for his advice and encouragement and Professor Stanley Peat, F.R.S., and Dr. J. R. Turvey for their continued interest. I am indebted to Dr. T. J. Painter who provided the authentic samples of sugar diethyl dithioacetals.

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