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THE POTASSIUM CHLORIDE-SOLUBLE CARRAGEENANS OF THE RED SEAWEED *Iridaea undulosa* B.

CARLOS A. STORTZ AND ALBERTO S. CEREZO*
Departamento de Química Orgánica, Facultad de Ciencias Exactas y Naturales (UBA), Pabellón 2, Ciudad Universitaria, 1428-Buenos Aires (Argentina)

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ABSTRACT

The potassium chloride-soluble fraction of carrageenan from the red seaweed *Iridaea undulosa* Bory contains both μ-like and λ-like carrageenans. These were partly separated by extraction with hot dimethyl sulfoxide, and characterized by methylation analysis, both before and after alkaline modification. In addition to regions composed of μ- or λ-type repeating units, both fractions contained regions of structural irregularity. Unusual units were found in these last regions, namely: (*a*) 2- and 6-linked galactose residues, (*b*) 3-linked, 6-sulfated and 2,6-disulfated residues, and (*c*) a small amount of branching with galacto- and xylo-pyranose units as nonreducing end groups. The determination of the changes in composition of the samples as a function of the number of methylation steps provided confidence in assignments of structural significance to minor components. These were characterized by g.l.c. and computerized g.l.c.–m.s. techniques under carefully chosen operating conditions, using two different sets of derivatives.

INTRODUCTION

The names κ- and λ-carrageenan were first given by Smith and Cook[1] to the fractions of carrageenan that were insoluble and soluble, respectively, in 0.125M potassium chloride. Later[2,3], it was found that λ-carrageenan defined in this way was not pure, and Rees *et al.*[4,5] separated two different polysaccharides after alkaline treatment of a carrageenan soluble in 0.4M potassium chloride. One was derived from the λ-carrageenan, but the other originated from a new type of carrageenan, μ-carrageenan, which has never been isolated in the native state.

Rees used the terms κ, λ, etc. for defined structures**, but the Norwegian school[6,7] extended the original, operational definition of Smith and Cook[1,2], and subdivided the fraction soluble in 0.125M potassium chloride into two parts,

*To whom correspondence should be addressed.
**For structural definition of these terms, see Table I.

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TABLE I

IDEALIZED REPEATING UNITS OF CARRAGEENANS[a]

Carrageenan	*3-Linked residue*	*4-Linked residue*
κ	β-D-galactopyranose 4-sulfate	3,6-anhydro-α-D-galactopyranose
ι	β-D-galactopyranose 4-sulfate	3,6-anhydro-α-D-galactopyranose 2-sulfate
μ	β-D-galactopyranose 4-sulfate	α-D-galactopyranose 6-sulfate
ν	β-D-galactopyranose 4-sulfate	α-D-galactopyranose 2,6-disulfate
λ	β-D-galactopyranose 2-sulfate (70%) and β-D-galactopyranose (30%)	α-D-galactopyranose 2,6-disulfate
ξ	β-D-galactopyranose 2-sulfate	α-D-galactopyranose 2-sulfate

[a]The definitions allow some structural variations (*e.g.* see ref. 26).

namely: (*a*) the fraction soluble at all concentrations of potassium chloride, which resembled Smith and Cook's λ-carrageenan most closely[1,2], and (*b*) the fractions precipitated at potassium chloride concentrations above 0.125M, which were described as "intermediate fractions". A semantic difficulty thus appeared in the use of the terms κ, λ, etc.

We have examined the carrageenans of the red seaweed *Iridaea undulosa*, soluble in 2M potassium chloride, for those defined types of structures forming part of, or coexisting, in the molecules. We also tried to detect the presence of unusual structural units which could form aperiodic sequences in these molecules.

Methylation analysis was considered the most fruitful approach to these problems, but the complete methylation of sulfated polysaccharides is difficult, due to steric hindrance and suppression of the ionization of neighboring hydroxyl groups by the ionized sulfate hemiester groups present. Also, the conventional approach of methylating to constant methoxyl content is unrealistic in these cases, because none of the sulfate groups is absolutely stable in concentrated alkali, and the methoxyl content never really stops going up.

An effort was made to solve these problems by determining the composition of the samples as a function of the number of methylation steps. This approach provides a useful indication of which fragments are genuine products of complete methylation, giving one confidence in assigning structural significance to minor components. The structural details deduced from the minor mono-*O*-methylated galactoses must nonetheless be considered tentative at present.

EXPERIMENTAL

General and analytical methods. — Galactose was analyzed by the phenol–sulfuric acid method[8], without previous hydrolysis of the polysaccharides. The galactose content was calculated by correcting for the presence of 3,6-anhydrogalactose, which was determined independently by the resorcinol method[9]. Sulfate was determined by the turbidimetric method of Dodgson and Price[10] and C-6

sulfate according to Rees[11]. Optical rotations were measured with a Perkin–Elmer 141 polarimeter using 0.4% solutions in 0.1M and 2.0M sodium chloride, 2M potassium chloride, and 7M urea. The solutions were equilibrated in the polarimeter tube for 18 h before the determinations. Reducing power was determined by the iodometric Somogyi method[12], with galactose as the standard. Viscosities were measured with a Cannon–Fenske viscosimeter at 20°; the Mark–Houwink equation was used to calculate molecular weights, using Masson constants[13]. Infrared spectra were recorded with a Perkin–Elmer 137B Infracord spectrophotometer using a film of the polysaccharide or a potassium bromide pellet.

G.l.c. analyses were performed in Hewlett–Packard Research Gas Chromatographs, models 5830 and 5840A, equipped with flame ionization detectors and glass columns (180 × 0.2 cm) containing 3% ECNSS-M on Gas-Chrom Q (100–120 mesh), operated isothermally at 180° with an injection chamber temperature of 210°. The liquid phase and the operating conditions were chosen after careful examination of different phases and programs[14]. Partially methylated sugars resulting from the acid hydrolysis of the permethylated carrageenans (45% formic acid for 16 h, 100°) were analyzed and identified by g.l.c. through the combined use of the acetylated alditol[15] and acetylated aldononitrile[14] derivatives. The use of the first procedure resolves all the partially methylated galactoses usually found in the hydrolyzates of methylated carrageenans except 2,3,6-tri-*O*-methyl- and 3,4,6-tri-*O*-methyl-galactose; 2,6-di-*O*-methyl- and 4,6-di-*O*-methyl-galactose; and 3-*O*-methylgalactose and the free sugar. When the acetylated aldononitrile derivatives are used, all the methylated galactoses are resolved except 4,6-di-*O*-methyl- and 3,6-di-*O*-methyl-galactose; and 2,3-di-*O*-methyl-, 2,4-di-*O*-methyl-, and 6-*O*-methyl-galactose[14].

Computerized g.l.c.–m.s. was performed on a glass column (120 × 0.2 cm) of 3% ECNSS-M on Gas-Chrom Q (100–120 mesh) at 180°, with helium as the carrier gas (30 mL/min), in a Varian Series 1440 gas chromatograph connected to a Varian MAT CH7A mass spectrometer.

Galactose and xylose were also determined by hydrolysis of the polysaccharides with 2M trifluoroacetic acid for 2 h at 121°, and conversion of the monosaccharide mixtures to the acetylated aldononitriles[14]. G.l.c. of these was performed with the 3% ECNSS-M column, operated isothermally at 190°.

Gel-permeation chromatography was performed on Sephadex G-100, with 7M urea as the eluant. The column was calibrated with dextrans of known weight-average molecular weight[16]; the mol. wt. exclusion limit of the column was about 40,000 (ref. 16).

The consumption of sodium periodate was determined by the spectrophotometric method[17]. Very little overoxidation was observed.

Extraction of carrageenan and preparation of "potassium chloride-soluble" carrageenan (A). — Whole carrageenan was extracted from *Iridaea undulosa* and purified as reported elsewhere[18]. Fraction A was precipitated from a 0.26% solution by adding potassium chloride to a concentration of 2M, separating the gel, dialyzing

the supernatant, concentrating it at room temperature, and freeze-drying (yield 18.6%). Carrageenan A was soluble at all concentrations of KCl. The yield of gel-forming carrageenans was 62.2%, the total yield, 80.8%.

Methylation of carrageenan A. — This was performed at −2° by the Haworth procedure[19] on carrageenan previously reduced with sodium borohydride. After each methylation the product was isolated and analyzed for methoxyl[20], sulfate, and 3,6-anhydrogalactose. It was also hydrolyzed with 45% formic acid (16 h, 100°), and the mixture of partially methylated sugars, derivatized as acetylated alditols and aldononitriles, was analyzed by g.l.c. Constant composition was obtained after five methylation sequences.

Fractionation of carrageenan A. — Carrageenan A (K^+ salt, 0.967 g) in water was passed through Amberlite IR-120 (H^+). The acidic effluent was neutralized with a 20% v/v pyridine solution, and after dialysis of the solution the pyridinium salt of the carrageenan was obtained by freeze-drying (0.886 g). This salt (0.697 g) was suspended in dimethyl sulfoxide (105 mL) and heated at 100°, with vigorous mechanical stirring. Aliquots were removed every 10 min, centrifuged, and analyzed for carbohydrates[8]. The amount of material solubilized became constant

TABLE II

YIELDS AND ANALYSES OF 2M KCl-SOLUBLE CARRAGEENAN AND OF SUBFRACTIONS SOLUBLE AND INSOLUBLE IN DIMETHYL SULFOXIDE[a]

Carrageenan	*A*[b]	*A*[c]	*As*	*Ai*
Yield %	13.0	18.6	57.7	38.8
Galactose (%)[d]	34 6	48.3	35.7	44.0
3,6-Anhydrogalactose (%)[d]	5.8	4.0	6.8	0.8
Sulfate (% SO_3K)	40.1	44 2	31.6	40.1
Gal:3.6-AnGal:SO_3K (molar ratio)	1.0:0.19:1 57	1:0.09:1.24	1 0.21·1.21	1:0.02 1.24
6-Sulfate (% SO_3K)[e]	3.0	7.3	5.6	8.4
Intrinsic viscosity (dl/g)	4.48	2.72	0 97	3 18
Xylose[f]	n.d.	n.d.	2.9	9.3
Periodate oxidation[g]	4.9	2.3	2.2	2.7
$[\alpha]_D$ in 0.1M NaCl	39.8	56.0	39.2	70.9
in 2M NaCl	n.d.	60.7	46.3	52 8
in 2M KCl	n d.	61.6	40.7	73.1
in 7M urea	n.d	61.9	40 3	72.9
Solubility in 2M KCl	sol.	sol.	sol	sol.
Mol. wt by viscosity[h]	64,000	42,000	18,000	48,000
by gel permeation[i]	n.d.	19,000	40,000	40,000
by reducing power[j]	n.d.	36,000	24,000	70,000

[a]The failure to obtain 100% recovery was attributed to tenaciously held moisture[3] [b]Fraction soluble in 2M KCl, obtained through stepwise precipitation[18]. [c]Fraction soluble in 2M KCl, obtained through bulk precipitation of the insoluble carrageenans at that concentration. [d]Percentages of the respective sugar residues. [e]Sulfate linked to primary hydroxyl. [f]Mols/100 mols galactose. [g]Average number of residues per mol of periodate consumed. [h]From the Mark–Houwink equation as determined by Masson[13]. [i]Using Sephadex G-100 in 7M urea, with non-sulfated carbohydrates as standards. [j]Measurements by the iodometric Somogyi method[12]

after 0.5 h, and heating was stopped after 1 h. The phases were separated, dialyzed to eliminate Me_2SO, and freeze-dried. The material insoluble in Me_2SO (Ai) was obtained in a yield of 38.8% (0.27 g), and As (soluble in Me_2SO) in a yield of 57.7% (0.402 g). The total yield was 96%. Analyses of As and Ai are given in Table II.

Alkali treatment of carrageenans A, As, and Ai. — The carrageenans (106.6 mg) were dissolved in water (53 mL) and reduced overnight with sodium borohydride (50 mg). Sodium hydroxide (3M, 26.5 mL) was added with sodium borohydride (100 mg) and the solution was heated for 5 h at 80°, until the production of 3,6-anhydro units was constant. The solution was then dialyzed and freeze-dried, producing 83.9, 68.5, and 73.2 mg, respectively of the alkali-treated carrageenans (89.9–91.7% yields, Table III). These carrageenans were fractionated with 0.4M potassium chloride and further with 2M potassium chloride as already mentioned[18]. Yields and analyses of the alkali-treated carrageenans and of the fractions isolated from them are given in Table III.

Methylation of carrageenans As and Ai, and of their products of alkaline treatment. — Carrageenans As, Ai, AsT_1, AsT_3, and AiT_3 (see Table III) were methylated by a four-step Haworth procedure[19] under the above mentioned conditions; only the temperature was changed (0° for As and Ai, room temperature for the others).

TABLE III

YIELDS AND ANALYSES OF THE ALKALI-TREATED CARRAGEENANS AND THEIR SUBFRACTIONS

Fraction	*Identifying characteristic*	*Yield (%)*	*Gal 3,6-AnGal·sulfate (molar ratio)*	*Residues/100 sugar units*	
				Sulfate	*3,6-AnGal*
A	Soluble in 2M KCl	18.6[a]	1:0.09:1.24	114	8
AT	Alkali treated	91.7[b]	1:0.44:1.37	95	31
AT_1	Insoluble in 0.4M KCl	33.0(34.7)[c]	1:0.97:1.25	63	49
AT_2	Insoluble in 0.4–2M KCl	5.8(6.1)[c]	n.d.	—	—
AT_3	Soluble in 2M KCl	56.3 (59.2)[c]	1:0.35:1.22	90	26
As	Soluble in Me_2SO	57.7[a]	1:0.21:1.21	100	17
AsT	Alkali treated	89.9[b]	1:0.40:0.95	68	29
AsT_1	Insoluble in 0.4M KCl	48.6(50.9)[c]	1:0.57:0.84	54	36
AsT_2	Insoluble in 0.4–2M KCl	6.0(6.3)[c]	1:0.59:1.34	84	37
AsT_3	Soluble in 2M KCl	40.9(42.8)[c]	1:0.26:0.92	73	21
Ai	Insoluble in Me_2SO	38.8[a]	1:0.02:1.24	122	2
AiT	Alkali treated	91.1[b]	1:0.43:1.54	108	30
AiT_1	Insoluble in 0.4M KCl	3.4(4.3)[c]	1:0.31:0.95	72	24
AiT_2	Insoluble in 0.4–2M KCl	5.1(6.5)[c]	1:0.56:1.60	103	36
AiT_3	Soluble in 2M KCl	70.5(89.2)[c]	1:0.48:1.55	105	32

[a]Yield from fractionation of parent carrageenan. [b]Yield from alkaline treatment. [c]Yield from fractionation of the alkali-treated carrageenans (in parentheses, percent of the total recovered).

TABLE IV

YIELDS IN THE SUCCESSIVE METHYLATIONS OF CARRAGEENAN A, AND METHOXYL, SULFATE AND 3,6-ANHYDROGALACTOSE VALUES FOR THE PRODUCTS

Methylation step	*Yield*[a] *(%)*	*Methoxyl (% OCH_3)*	*Sulfate (% SO_3Na)*	*3,6-Anhydrogalactose (%)*[b]
—	—	0	30.6	5.8
1	86.6	8.1	26.5	9.6
2	61.5	13.2	28.0	10.3
3	99.7	12.6	26.7	8.0
4	100.0	13.2	28.7	6.6
5	95.2	15.2	25.9	5.7

[a]Yield in the step shown; the overall yield was 50.5%. [b]Calculated for the sugar residue

RESULTS

Carrageenan A was obtained from the supernatant from the bulk precipitation of the products insoluble in 2M KCl. Its yield and composition, and some of its properties, are given in Tables II and III. The i.r. spectrum suggested the presence of different types of sulfate groups, mainly linked to secondary, equatorial hydroxyls.

Methylation analysis of carrageenan A. — Analytical data for the product obtained after each step of methylation are given in Table IV, and the percentages of the methylated fragments from each product are given in Table V. The results show a loss of sulfate residues concomitant with the production of 3,6-anhydro units, consistent with the alkaline conversion of 4-linked, 6-sulfated and/or 4-linked, 2,6-disulfated galactose residues. There was subsequently some loss of 3,6-anhydrogalactose units during the methylation.

The composition of the permethylated derivative of carrageenan A in terms of partially methylated, acid-stable sugars is given in Table V, and in Table VIII this is expressed as a distribution of strucural units in the original carrageenan.

Alkaline treatment of carrageenan A. — The product (AT) obtained after alkaline treatment of carrageenan A had the composition shown in Table III. Comparison of this composition with that of the original carrageenan (A) shows that there was an increase in the percentage of 3,6-anhydrogalactose units (23 units/100 total units) with a concomitant decrease of sulfate groups (19 units/100 total units).

Carrageenan AT was fractionated with potassium chloride into two major fractions, namely: (*a*) that precipitated at 0.4M KCl (AT_1, 33.0%) and (*b*) that soluble in 2M KCl (AT_3, 56.3%). Only a small amount of material (AT_2, 5.8%) was precipitated at KCl concentrations between 0.4 and 2M. Analyses for the potassium chloride insoluble (AT_1) and soluble (AT_3) carrageenans are given in Table III. The composition of AT_1 and the i.r. spectrum (Fig. 1) suggest a structure of the hybrid κ/ι-carrageenan type, and consequently the presence of μ/ν-like structures

TABLE V

ACID-STABLE PARTIALLY METHYLATED SUGARS PRODUCED BY THE METHYLATION AND HYDROLYSIS OF CARRAGEENAN A

Methylation step	*Mole percent of the sugar having methyl groups at the positions indicated*														
	Galactose														*Xylose*
	2,3,4,6	*2,4,6*	*2,3,6*	*3,4,6*	*2,3*	*2,4*	*2,6*	*4,6*	*3,6*	*2*	*3*	*4*	*6*	*None*	*2,3,4*
1	0.4	0.6	0.3	0.1	—1.6—		——34.7——		—	9.6	6.1	4.5	16.6	25 5	—
2	0.5	1 5	1.3	0.4	1.9	1.8	37.5	9.3	1.2	4 0	9.2	8.7	8.7	12.2	1.7
3	0.7	2.5	2.0	0.3	2 1	2 0	37.2	15.3	1 5	3.1	10.4	7.6	6.3	7.0	2.1
4	2.2	2.5	2.9	3.2	2.6	1.7	39.0	17.1	1.5	4.1	11.8	4.5	2.0	1.9	2.9
5	3.1	3.5	1.3	2.6	3.2	4.2	40.3	19 4	1.1	3.1	7.8	4.0	1.6	1.0	3.8

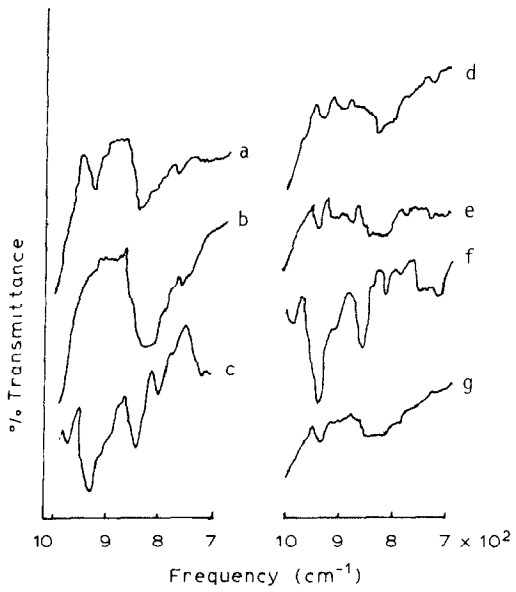

Fig. 1. Infrared spectra (700–1000 cm^{-1}) of carrageenans As (a), Ai (b), AT_1 (c), AT_3 (d), AiT_3 (e), AsT_1 (f), and AsT_3 (g)

in carrageenan A. A shoulder at 820 cm^{-1} in the i.r. spectrum suggests that minor amounts of C-6 sulfate groups survived the alkaline treatment, and this is consistent with the finding of 2,4-di-*O*-methyl- and 4-*O*-methyl-galactose in the hydrolyzate of the permethylated A (Table V). This result suggests the existence of 3-linked, 6-sulfated units in A and AT_1.

The molar ratio G:A:S (Gal:3,6-AnGal:SO_3K) of AT_3 (Table III) suggests that a substantial portion of the 3,6-anhydro units are 2-sulfated, and that they were formed from 4-linked, 2,6-disulfated residues. The i.r. spectrum (Fig. 1) indicated the prominence of secondary, equatorial sulfate groups but also the presence of significant amounts of sulfate linked to other positions.

Fractionation of carrageenan A. — Stirring of the pyridinium salt of carrageenan A in Me_2SO at 100° produced partial solubilization. Analyses for both fractions, Me_2SO-soluble carrageenan (As, 57.7%) and Me_2SO-insoluble carrageenan (Ai, 38.8%), and some of their properties, are shown in Table II. The data suggest a fractionation based partially upon composition, but mainly upon molecular weight, with little desulfation or degradation.

Carrageenan Ai. — This showed (Table II) a molar G:A:S ratio in agreement with Rees' definition of a λ-carrageenan. The product also contained 3.3% of xylose, and its i.r. spectrum was characteristic of a λ-carrageenan (Fig. 1).

Methylation of carrageenan Ai. — The composition of the partially methylated products after each step of the methylation is given in Table VI. It is noteworthy that 5% of the units are actually branching points, possibly carrying

TABLE VI

ACID-STABLE PARTIALLY METHYLATED SUGARS PRODUCED BY THE METHYLATION AND HYDROLYSIS OF CARRAGEENANS As AND Ai

Carrageenan and methylation step	*Mole percent of the sugar having methyl groups at the positions indicated*														
	Galactose														*Xylose*
	2,3,4,6	*2,4,6*	*2,3,6*	*3,4,6*	*2,3*	*2,4*	*2,6*	*4,6*	*3,6*	*2*	*3*	*4*	*6*	*None*	*2,3,4*
As 1	—	——— 2.8 ———			2.3	1.6	29.4	7.5	1.7	10.3	5.2	9.0	8.0	21.8	0.5
2	0.8	2.2	—— 2.0 ——		4.7	3.0	34.3	17.4	3.2	2.7	4.4	9.2	3.7	9.7	2.7
3	1.9	2.7	—— 3.2 ——		5.7	5.8	36.1	15.7	5.3	3.3	4.1	8.9	2.3	1.3	3.7
4	1.4	5.7	—— 3.9 ——		0.9	2.0	41.1	16.9	6.7	2.4	6.1	4.7	4.9	0.1	3.3
Ai 1	—	—— 2.0 ——		—	0.8	1.0	3.0	4.7	0.5	2.7	5.2	8.4	12.5	58.7	0.5
2	0.1	——— 4.8 ———			1.1	1.7	6.1	14.6	2.0	2.2	14.7	14.2	12.0	25.2	1.4
3	0.9	3.1	1.4	2.2	2.2	5.3	9.3	36.0	4.7	1.6	8 9	9.7	8.1	5.5	1.2
4	tr.	5.3	0.8	3.2	1.8	4.2	10.9	43.3	3.5	1.0	6.7	8.8	5.3	0.1	5.0

side-stubs of single xylopyranose residues. Only traces of tetra-*O*-methylgalactose were found, in agreement with the high molecular weight. The percentage of 3,6-anhydrogalactose (0.8%) in Ai increased to 8.2% in its permethylated derivative, showing that most of the 4-linked, 6-sulfated and 2,6-disulfated units were transformed into 3,6-anhydro residues during the methylation.

Alkaline treatment of carrageenan Ai. — The product AiT, obtained in 91.1% yield, had a G:A:S ratio of 1:0.43:1.54 (Table III). Its i.r. spectrum indicated more C-2-equatorial and C-6-primary sulfate groups than C-4-axial ones. It also indicated the presence of major amounts of 3,6-anhydro, C-2-axial sulfate, in agreement with the molar ratio of the components. The formation of the 2-sulfated, 3,6-anhydrogalactose units from the 4-linked, 2,6-disulfated residues in the alkaline treatment was clear from a comparison of the i.r. spectra of carrageenans Ai and AiT.

Carrageenan AiT when fractionated with potassium chloride gave only one major fraction (AiT_3, 70.5%) soluble in 2M KCl, together with small amounts of products precipitated at 0.4M KCl (AiT_1, 3.4%), and between 0.4 and 2.0M KCl (AiT_2, 5.1%).

The carrageenan AiT_1 had a sulfate content consistent with its gelation properties, but a 3,6-anhydrogalactose percentage much lower than expected for these properties (Table III). Fraction AiT_2 had a G:A:S ratio of 1:0.56:1.60. The content of sulfate compared to that of AiT_1, was 42% greater, and this could explain its higher solubility in spite of the increase in 3,6-anhydrogalactose content (35%). The high content of sulfate suggests that both sugar units carry this group. The "carrabiose content" [carrabiose = β-D-Gal-(1→4)-3,6-AnGal] is 72% but the polysaccharide is still soluble in 0.4M KCl.

The major fraction AiT_3 had a G:A:S ratio of 1:0.48:1.55 (Table III). This composition is similar to that of AiT_2, although the solubility is higher. Thus the carrabiose content (65% in this case) is not the only factor that determines the solubility. It is also neccessary to consider the sequence of the carrabiose units, which governs the distribution of "kinking" residues along the chain. The i.r. spectrum of AiT_3 (Fig. 1) showed the absence of 3-linked, 4-sulfated units and the presence of major amounts of secondary equatorial and axial (on a 3,6-anhydro ring) sulfate groups and of minor amounts of sulfate half-ester groups attached to primary hydroxyls in 3-linked units.

Methylation analysis of carrageenan AiT_3. — The composition of the permethylated derivative is given in Tables VII and VIII. It was noteworthy to find 3-*O*-methylgalactose (2.7%), which is usually supposed to be produced from 4-linked, 2,6-disulfated units; and also 2,3-di-*O*-methylgalactose, which is usually formed from 4-linked, 6-sulfated units. The presence of these partially methylated sugars in an alkali-treated carrageenan seems to indicate that some 4-linked galactose units also act as branching points through C-6, or possibly that some units in the polysaccharide are linked through C-6.

Unusual residues in AiT_3 are shown by the presence, in the permethylated

TABLE VII

ACID-STABLE PARTIALLY METHYLATED SUGARS FROM PERMETHYLATED CARRAGEENANS AsT_1, AsT_3, AND AiT_3

Carrageenan	*Mole percent of the sugar having methyl groups at the positions indicated*[a]														
	Galactose														*Xylose*
	2,3,4,6	*2,4,6*	*2,3,6*	*3,4,6*	*2,6*	*4,6*	*3,6*	*2,4*	*2,3*	*6*	*2*	*3*	*4*	*None*	*2,3,4*
AsT_1	tr.	8.7	tr.	8.4	50.7	—— 6.4 ——		—— 16.4 ——		4.1	tr.	tr.	3 2	—	2.1
AsT_3	3 9	5.4	2.3	1.9	7.7	36.0	9.4	2.9	—— 12.5 ——		tr.	3.8	11 5	1.4	1.3
AiT_3	3.4	5.6	4.0	3 6	5.8	33.7	12.5	1.7	3 5	6.2	1.9	2.7	11.5	1.5	2.5

[a]Percentages lower than 1% are given as "trace" (tr.).

TABLE VIII

THE COMPOSITION OF THE CARRAGEENANS STUDIED

Carrageenans	*Percentage of the sugar units having sulfate at the positions indicated*														
	3-Linked galactose							*4-Linked galactose*				*3,6-AnGal*[a]	*Other units*		
	None	*2*	*4*	*6*	*2,6*	*2,4*	*4,6*	*None*	*2*	*6*	*2,6*		*Gal*		*Xyl*
													2L[b]	*T*[c]	*T*[c]
A[d]	2.9	16.2	33.6	3.5	3.4	1.4	2.6	1.1	tr.	3.3	6.5	15.9	2.2	2.6	3.2
As[d]	4.0	11.8	28.6	1.4	3.3	3.4	1.7	2.7[e]	4.6	—— 17.5 ——		17.6	2 7[e]	tr.	2.4
Ai[d]	4.2	34.4	8.7	3.3	7.0	4.2	tr.	tr.	2.7	—— 25.5 ——		2.0	2.6	tr.	3.8
AsT_1	5.4	4.0[e]	31.6	9.5[f]	2.0	3.2	tr.	tr.	4.0[e]	9.5[f]	tr.	36.3	5.2	tr.	1.3
AsT_3	4.3	28.4	6.1	2.3	9.0	9.8[f]	tr.	1.8	7.4	9.8[f]	3.0	20.6	1.5	3.1	1.0
AiT_3	3.8	22.8	4.0	1.1	7.8	4.2	1.3	2.7	8.5	2.4	1.8	32.4	2.4	2.3	1.7

[a]3,6-Anhydrogalactose plus its 2-sulfated derivative. [b]2-Linked, non-sulfated. [c]Non-sulfated terminal units. [d]Corrected for cyclization produced during the methylations. [e]The figure shows the contributions of both sugars, mainly the 4-linked unit. [f]The figure shows the contributions of both sugars, mainly the 3-linked unit.

derivative, of 2,3,6-tri-*O*-methylgalactose and 3,4,6-tri-*O*-methylgalactose. The presence of significant amounts of 3,6-di-*O*-methylgalactose, in spite of the supposed alkali lability of 4-linked, 2-sulfated residues, is also noteworthy. The 2,4-di-*O*-methyl- and 4-*O*-methyl-galactoses could be derived from 3-linked units sulfated on C-6, or on C-6 and C-2, respectively, in agreement with the i.r. spectrum (Fig. 1).

Carrageenan As. — This had a G:A:S ratio (Table II) typical of a μ/ν-carrageenan. It contained 0.8% of xylose, and its i.r. spectrum (Fig. 1) showed bands corresponding to sulfate groups linked to axial O-4 in the galactose units and to axial O-2 in the 3,6-anhydro units.

Methylation analysis of carrageenan As. — The compositions of the partially and completely methylated carrageenans are given in Tables VI and VIII. The 2,3,4-tri-*O*-methylxylose found as the only methylated derivative of that sugar suggested the presence of xylopyranose nonreducing end-groups (3.3%), and consequently a similar percentage of branching points in the central chain.

Alkaline treatment of carrageenan As. — The product (AsT) obtained with 89.9% yield had a G:A:S ratio of 1:0.40:0.95 (Table III). The i.r. spectrum showed the presence of axial sulfates in galactose and 3,6-anhydrogalactose units. A minor absorption was also observed that suggested the presence of C-6 sulfates, which could not be situated on 4-linked units.

The carrageenan AsT was fractionated with potassium chloride solutions to produce two major fractions and a minor one, namely: (a) insoluble in 0.4M KCl (AsT_1, 48.6%), (b) insoluble in 0.4–2.0M potassium chloride (AsT_2, 6.0%), and (c) soluble in 2M potassium chloride (AsT_3, 40.9%). Analyses of the three fractions are given in Table III.

In the major fraction AsT_1, which had a G:A:S ratio of 1:0.57:0.84, the ratio galactose:sulfate is that expected for gel-forming carrageenans but the 3,6-anhydrogalactose content is low for these products. Methylation analysis (see later) showed that the sulfates were distributed in a manner consistent with the solubility properties and the i.r. spectrum (Fig. 1). The latter additionally indicated that most of the 3,6-anhydro units were sulfated, which is consistent with the solubility of the polysaccharide in 0.1M KCl.

The carrageenan AsT_2 had a G:A:S ratio of 1:0.59:1.34. The ratio G:A is the same as in AsT_1, but the percentage of sulfate is increased by 60%, consistent with the higher solubility of AsT_2.

The other major fraction, AsT_3, had a G:A:S ratio of 1:0.26:0.92, showing a high 3,6-anhydrogalactose and a low sulfate content for KCl-soluble carrageenans. The i.r. spectrum (Fig. 1) indicated the presence of major amounts of equatorial sulfate groups, with minor percentages of galactose 4- and 6-sulfates (the latter not present as 4-linked units), and the absence of axial C-2 sulfates on the 3,6-anhydrogalactose units.

Methylation analysis of carrageenans AsT_1 and AsT_3. — The compositions of the permethylated derivatives are given in Table VII and the interpretation of the

results in Table VIII. Fraction AsT_1 contained major amounts (50.7%) of 3-linked, 4-sulfated units and a significant percentage (8.7%) of 3-linked, non-sulfated residues. The presence of these units is in agreement with the solubility characteristics of AsT_1. Unusual 3-linked units were shown by the presence of significant amounts (16.7%) of 2,4-di-*O*-methylgalactose. Most of the parent units are probably 3-linked, 6-sulfated residues, as indicated by the i.r. spectrum (Fig. 1), although some may be branching points. Similarly, the small amount (3.2%) of 4-*O*-methylgalactose may be derived from 3-linked, 2,6-disulfated residues. The 6-*O*-methylgalactose is postulated to be formed from 3-linked, 2,4-disulfated residues on the grounds that no sulfate groups were ever found at position 3.

The 3,6-di-*O*-methylgalactose suggests the presence of 4-linked, 2-sulfated units. These units, in an alkaline medium, could in principle form the 2,3-epoxide and then be converted into other products. The actual amount of 4-linked, 2-sulfated units in AsT_1 could therefore be higher than that shown in Table VIII. However, comparison of the compositions of methylated As, AsT_1, and AsT_3 (Tables VI and VII) shows similar amounts of 3,6-di-*O*-methylgalactose, suggesting that alkaline treatment of As has not destroyed any of the 4-linked, 2-sulfated units.

Methylated AsT_3 produced a pattern of partially methylated galactoses qualitatively similar to that of methylated AiT_3 (Table VII). The major structural unit in AsT_3 was the 3-linked, 2-sulfated residue (36.0%); these units, together with the 3-linked, non-sulfated (5.4%) and 3-linked, 4-sulfated (7.7%) residues are usually found in carrageenans that are soluble in high concentrations of KCl. As in AiT_3, it is noteworthy to find 3-*O*-methylgalactose in an alkali-treated product. Unusual residues are shown by the presence of 2,3,6-tri-*O*-methylgalactose (2.3%) and 3,4,6-tri-*O*-methylgalactose (1.9%). The presence of significant amounts of 3,6-di-*O*-methylgalactose (9.4%) is noteworthy, considering the supposed alkaline lability of the 4-linked, 2-sulfated residue. The 2,4-di-*O*-methylgalactose (2.9%) and the 4-*O*-methylgalactose (11.5%) could be derived from 3-linked units, sulfated at C-6 and at C-2 and C-6, respectively, in agreement with the i.r. spectrum (Fig. 1) of AsT_3.

DISCUSSION

The "potassium chloride-soluble" carrageenan A has a composition and optical rotation (Table II) similar to those of other KCl-soluble carrageenans[21]. The molar ratio of galactose to sulfate (2:2.5) is close to that defined for the "ideal" λ-carrageenan[4,22] (Table I), in agreement with the i.r. spectrum, although the polysaccharide contained a small but significant percentage of 3,6-anhydrogalactose.

When carrageenan A was submitted to alkaline treatment and further fractionated with potassium chloride, it yielded two products whose solubility behavior suggested that the original polysaccharide contained molecules having the μ- and λ-structures[5]. This is consistent with the fractionation brought about by dimethyl sulfoxide (see later).

The distribution of structural units in carrageenan A is given in Table VIII. It is different from that found in carrageenans from the same seaweed which were precipitated by 0.70–1.05M potassium chloride[23] and similar to, but more complicated than, that of the carrageenans precipitated by 1.55–1.65M potassium chloride[23]. The distribution is in agreement with the previously mentioned μ-like and λ-like structures. The methylation analysis of A (Table V) shows the presence of significant amounts of previously unknown structural units (Table VIII). The corresponding partially methylated galactoses were also found in the permethylated derivatives of the above mentioned "intermediate fractions" of the same carrageenan of *Iridaea undulosa*, but they were not discussed[23]. The new units were confirmed in the studies of the carrageenans soluble (As) and insoluble (Ai) in dimethyl sulfoxide, and in the analysis of the products obtained from them by alkaline-borohydride treatment, and will be discussed later.

The carrageenan insoluble in dimethyl sulfoxide (Ai) has the composition (Table II) and i.r. spectrum (Fig. 1) of a λ-carrageenan; it contains *ca.* 65% of units defined as components of λ-carrageenans, but it also contains substantial amounts (~15%) of units regarded as components of μ-carrageenans. The fact that the alkaline treatment of Ai produced 8.5% of potassium chloride-insoluble products suggests that μ-carrageenan can exist as a separate polysaccharide. If this is true, Ai is not a heterogeneous block copolymer, with μ-like and λ-like blocks, or a polysaccharide composed of μ- and λ-units with "unusual" residues interspersed between them, but a mixture of two carrageenans, one of them containing μ-blocks and the other λ-blocks, and both molecules complemented with "unusual" residues. The same situation was found in the dimethyl sulfoxide-soluble carrageenan As (see later).

The alkaline treatment of Ai produced in quantitative yield a carrageenan (AiT) that when fractionated with potassium chloride solutions (Table III) gave 89.2% of a soluble product (AiT_3). This further supports the conclusion that Ai was composed mainly of λ-carrageenan.

Fraction AiT_3 had a carrabiose content of 65%, but it failed to gel, in contrast to AiT_1 (Table III) which, despite having a carrabiose content of only 47%, was insoluble in 0.4M KCl. It is not possible, at present, to determine whether the solubility is due to the carrabiose units being non-contiguous, or whether the "unusual" structural units (see later) preclude the formation of a gel network.

The carrageenan soluble in dimethyl sulfoxide (As) had the composition (Table II) and i.r. spectrum (Fig. 1) of a μ-carrageenan. Its low molecular weight (Table II) is consistent with previous findings on the carrageenans of *Iridaea* sp.[18], and with the fact that μ-carrageenans are regarded as biological precursors of κ-carrageenans. Fraction As is constituted of major amounts (Table VIII) of structural units defined as components of μ- and λ-carrageenans. On alkali treatment it produced both potassium chloride-insoluble (AsT_1 and AsT_2) and -soluble (AsT_3) carrageenans (Table III), showing that, like Ai, As is a mixture of μ-like and λ-like carrageenans. This is consistent with the hypothesis that the

fractionation in dimethyl sulfoxide depended mainly on differences in molecular weight.

Fraction AsT_1 has the composition of an ι-carrageenan with a low percentage of 3,6-anhydro units, and a carrabiose content of 72.6% (Table III). This content is in agreement with the percentage of ι-structure (67.9%, Table VIII), and would explain the insolubility in potassium chloride solutions; the rest of the molecule contains "unusual" units which would possibly affect the range of solubility.

Fractions AsT_3 and AiT_3 have a new type of chain resulting from the action of alkali upon a partial λ-structure. This λ-structure comprised about 41% and 64% of the molecules of the λ-components of As and Ai, respectively, as judged by the carrabiose contents of AsT_3 and AiT_3.

The main characteristic of the AsT_3 and AiT_3 type of carrageenans is the complete solubility in potassium chloride in spite of their high carrabiose contents. For these materials the relationship between 3,6-anhydrogalactose content and the gelling properties is not maintained.

"Unusual" structural units. — Tables V, VI, and VII show the presence, in the permethylated carrageenans, of partially methylated galactoses derived from the following structural units which were not previously detected:

(*a*) Nonreducing end-groups. The presence of nonreducing end-groups of xylopyranose (in all studied carrageenans) and galactopyranose (in AiT_3 and AsT_3) indicated a small amount of branching in the linear backbone, possibly by single-unit stubs. Xylose has previously been detected in carrageenans[24], and its possible role as a lateral chain was mentioned, but this possibility was discarded in favor of the assumption of a contaminating xylan[24]. Nonreducing xylosyl end-groups were also suggested in the polysaccharides of *Phyllimenia hieroglyphica* and *Pachymenia hymantophora*[25]. The presence of these lateral chains would increase the solubility of the polysaccharides.

(*b*) 2-Linked, non-sulfated units. These units would produce a turn in the direction of the backbone, disturbing the formation of ordered aggregates. Alternatively, the 3,4,6-tri-*O*-methylgalactose observed may indicate terminal, 2-sulfated units.

(*c*) 4-Linked, 2-sulfated units. This kind of residue has been found in the "soluble" carrageenans of *Gigartina canaliculata*[26] and *Gigartina chamissoi*[26], and in the whole carrageenan of *Gigartina atropurpurea*[26]. It has been suggested that the percentages found are lower than those present in the original molecules, owing to the possible destruction of the unit by alkali. Nevertheless, Table VIII shows that after alkali treatment and methylation the percentages of 4-linked, 2-sulfated units in As and Ai have not diminished. Manual integration of the gas-liquid chromatograms of the methanolysis products of the methylated ξ-fraction from *Gigartina canaliculata* gave a molar ratio of 4,6-di-*O*-methyl- to 3,6-di-*O*-methyl-galactose of 5.5:4.5. This is in agreement with the proposed structure for the ξ-carrageenan, but not with extensive elimination of sulfate to form the 2,3-epoxide, which could then be converted into other products[26].

(*d*) 3-Linked, 6-substituted units. The lateral chains indicated by the terminal xylo- or galacto-pyranose residues could be linked to the backbone through C-6 of a 3-linked unit as previously suggested[26]. This would be consistent with the finding of 2,4-di-*O*-methylgalactose and the xylose derivative in similar amounts in the methylated derivatives of Ai and AiT_3. The fact that in AsT_1 and AsT_3 the amount of the dimethylated galactose is higher than that of the terminal units suggests that in some cases the C-6 could be sulfated.

(*e*) The presence of 2,3-di-*O*-methylgalactose in permethylated AiT_3 and possibly in other permethylated derivatives of the alkali-treated carrageenans may indicate the existence of 6-linked, 4-sulfated units. The flexibility of the 1→6 linkage would increase the solubility of the molecule[27], disturbing the formation of aggregates.

The following, additional suggestions of new structural units are based on the detection of 4-*O*-methyl- and/or 6-*O*-methyl-galactose in the permethylated derivatives of the carrageenans. Even though the amounts determined are significant (Tables V, VI, and VII), the conclusions are only tentative due to the possibility that these derivatives may be products of incomplete methylation.

(*f*) If the monomethylated units in Tables V, VI, and VII are to be explained in terms of a repeating structure, the 4-*O*-methylgalactose would arise from a 3-linked residue disulfated on C-2 and C-6 or from a 3-linked, monosulfated branching point. Units of the first type, namely 3-linked galactopyranosyl 2,6-disulfate residues, have been found in the polysaccharide of *Pachymenia hymantophora*[25]. The 6-*O*-methylgalactose suggests the existence of 3-linked, 2,4-disulfated units and/or 4-linked, 2,3-disulfated units. Until now, no sulfates have ever been found at position 3 in the galactose residues of carrageenans.

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