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The systems of carrageenans from cystocarpic and tetrasporic stages from *Iridaea undulosa*: fractionation with potassium chloride and methylation analysis of the fractions

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ABSTRACT

The carrageenans produced by the cystocarpic and tetrasporic stages from *Iridaea undulosa* were fractionated by potassium chloride precipitation and analyzed. The cystocarpic system is composed of similar amounts of a gelling carrageenan with κ/ι-characteristics, and a soluble, partially cyclized, μ/ν-carrageenan. The tetrasporic system is composed mainly of a λ-carrageenan but also with a small proportion of a galactan sulfate containing L-galactose. The structures of the components of both were examined by methylation analysis. Correlation with previous data from carrageenans of the same and different sources allows us to investigate some of the problems in the chemistry of these polysaccharides, namely: (a) the formal pattern of the systems biosynthesized by each stage of the seaweed and the relationships between the components, (b) general relationships between the primary structure and solubility in potassium chloride solutions, and (c) the solubility of carrageenan mixtures versus that of "pure" compounds in these solutions.

INTRODUCTION

The system of carrageenans from the red seaweed *Iridaea undulosa* has been studied[1–4] with material extracted from unsorted samples. Over the last decades, it has been shown that members of the family Gigartinaceae yield different carrageenans from the karyologically different generations[5,6]. Careful studies of the carrageenans from the cystocarpic and tetrasporic stages from *Gigartina skottsbergii*[7] showed that the cystocarpic samples produce a two-product system composed of a soluble, partially cyclized μ/ν-carrageenan and a gel-forming κ/ι-carrageenan, while the tetrasporic sample biosynthesize a system of disperse λ-carrageenans precipitable at "intermediate" concentrations of potassium chloride[7]. Their structures were demonstrated by methylation analysis and ^{13}C and ^{1}H NMR

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spectroscopy[8,9]. Systems similar to those mentioned above were also found in *Chondrus crispus*[5].

As the interpretation of the results produced with unsorted samples was restricted by the complexity of the carrageenan mixtures, this paper reports the analysis and characterization of the systems of carrageenans from cystocarpic and tetrasporic samples of *Iridaea undulosa*, and the structural analysis by methylation of the seven fractions isolated. The correlation between primary structures and solubility in potassium chloride solutions and the solubility of carrageenan mixtures versus that of "pure" compounds are discussed.

RESULTS

Extraction and fractionation of cystocarpic plants.—The cystocarpic plants (female gametophytes) were extracted with water at room temperature, yielding a polysaccharide (CWS-1). The residue was reextracted, giving carrageenan CWS-2. The pooled alcoholic solutions yielded carrageenan CWS-F.

A highly hygroscopic product was obtained from the 2-propanol supernatant. This product was mostly inorganic with only 2–3% carbohydrate content and was therefore discarded.

The residue was reextracted, yielding carrageenan CWS-3. After completion of the extractions at room temperature, the residue was extracted with boiling water to give the polysaccharide CHS. A second hot-water extraction did not yield any 2-propanol-precipitable product.

Yields and analyses of the products are shown in Table I. All the carrageenans sequentially extracted are similar (Table I). Even the hot water-extracted carrageenan has the same characteristics. Thus, carrageenans CWS-1, CWS-2, CWS-3 and CWS-F were dissolved together in water, and the product purified by centrifugation. The lyophilized carrageenan (C) was fractionated with potassium chloride.

TABLE I

Yields and analyses of carrageenans extracted from cystocarpic and tetrasporic plants from *Iridaea undulosa*

Product	Yield (%)	Gal (% anh.)	3,6-AnGal (% anh.)	Sulfate (% SO_3K)	Molar ratio Gal:AnGal:S
CWS-1	33.5	48.0	20.8	35.8	1:0.48:1.02
CWS-2	18.3	47.6	21.3	35.3	1:0.50:1.01
CWS-3	3.9	43.2	21.7	35.4	1:0.57:1.12
CWS-F	1.1	39.6	17.2	30.4	1:0.49:1.05
CHS	6.1	47.7	20.6	40.3	1:0.49:1.15
TWS-1	27.1	51.6	0.0	40.3	1:0.00:1.06
TWS-2	10.8	45.6	0.2	38.9	1:0.00:1.16
TWS-3	3.6	45.1	0.2	37.4	1:0.00:1.13
TWS-F	6.0	50.0	0.4	32.6	1:0.01:0.89
THS	3.6	46.9	0.2	33.9	1:0.00:0.98

TABLE II

Yields and analyses of the carrageenans obtained by KCl fractionation of the total cystocarpic (C) and tetrasporic (T) carrageenans from *Iridaea undulosa*

Precipitation range (M KCl)	Yield [a] (%)	Molar ratio Gal:AnGal:S	6-S [b] per Gal	2,6-diS [a] per Gal	Molar ratio AnGal:6S:2,6S (per sugar)	$[\alpha]_D$ (°)
C		1:0.49:1.04	0.14	0.27	0.33:0.10:0.18	
C_1 0.5–0.7	41.7 (43.4)	1:0.62:0.99	0.08	0.19	0.38:0.05:0.12	+63.8
C_2 0.9–1.0	1.8 (1.9)	1:0.56:1.04	0.18	0.07	0.36:0.11:0.05	+63.0
C_3 1.3–1.4	1.1 (1.1)	1:0.52:0.97	0.00	0.28	0.34:0.00:0.18	+68.4
C_4 2 (sol)	51.4 (53.5)	1:0.46:1.11	0.17	0.29	0.32:0.11:0.20	+44.3
T		1:0.00:1.26	0.00	0.50	0.00:0.00:0.50	
T_1 1.0–1.2	72.3 (85.6)	1:0.00:1.17	0.02	0.43	0.00:0.02:0.43	+78.4
T_2 1.2–1.4	3.6 (4.3)	1:0.01:1.37	0.02	0.50	0.01:0.02:0.50	+71.6
T_3 2 (sol)	8.6 (10.2)	1:0.02:0.73 [d]	0.00	0.19	0.02:0.00:0.18	+11.2

[a] In parentheses, percent of the total recovered. [b] 4-Linked, 6-sulfated units. [c] 4-Linked, 2,6-disulfated units. [d] The galactose is 30% in the L-form and 70% in the D-form.

Yields and analyses of the fractions are shown in Table II. No classic κ-fraction (precipitating below 0.125 M KCl) appears, but two major fractions, an "intermediate" (C_1) and a "soluble" one (C_4), represent the gametophytic carrageenan. Both these fractions and the minor ones C_2 and C_3 present similar analytical characteristics, though the 3,6-anhydrogalactose content is inversely proportional to the KCl solubility. All fractions present a molar ratio of galactose to sulfate close to one. The optical rotation of the gelling fractions is within a narrow range (63.0–68.4°), which is different from the value observed for the soluble fraction C_4 (44.3°).

Extraction and fractionation of tetrasporic plants.—The same procedure of sequential water extraction was used with tetrasporic samples. Three water extractions yielded the products TWS-1, TWS-2, and TWS-3, respectively. Their yields and analyses are given in Table I. Also, an additional product that precipitated from the 75% 2-propanol of TWS-1 was obtained (TWS-F). Its characteristics are also given in Table I, as are those from the hot water-extracted product THS.

These products contain, at most, only trace amounts of 3,6-anhydrogalactose, i.e., the products classically known as λ-carrageenans. The results and analysis of the potassium chloride fractionation of carrageenan T (i.e., the sum of those extracted at room temperature) are shown in Table II. Arbitrary fractionation of the product precipitating between 1.0 and 1.4 M potassium chloride showed a main fraction (T_1) with analytical characteristics of a λ-carrageenan, but a precipitation behavior of an "intermediate" fraction, and a similar fraction (T_2) isolated in a smaller amount. Both fractions have mostly 2,6-disulfated 4-linked units (Table II). The "soluble" fraction (T_3) represents a product (Table II) with a low degree of sulfation and a low optical rotation. This is due to the fact this fraction contains both D- (70%) and L-galactose (30%).

TABLE III

Composition of methylated galactoses (molar ratio) after methylation of carrageenans C_1 and T using different periods of reaction with methylsulfinyl carbanion [a]

Methyl group on	C_1		T		
	10 min	40 min	10 min	40 min	100 min
2,3,4,6-Tetra	0.2	0.1			
2,4,6-Tri	0.6	0.6	3.6	1.5	1.3
2,3,6-Tri	1.2	0.7	3.7	3.2	1.0
2,3,4-Tri	0.9	0.2			
2,6-Di	84.4	89.4	3.3	2.8	3.0
4,6-Di	↑ 3.3	↑ 2.4	39.5	↑ 55.3	51.9
3,6-Di	↓	↓	1.6	↓	3.2
2,3-Di	2.9	2.1	2.4	2.2	2.3
2,4-Di	1.0	0.5	1.7	2.3	2.4
6-Mono	0.4	0.2	3.5	4.1	6.0
2-Mono	0.8	1.3	2.4	0.9	0.5
3-Mono	4.4	2.5	17.2	18.6	21.9
4-Mono			4.2	3.3	4.8
Gal			16.9	5.8	1.3

[a] For comparison purposes, even peaks present as traces (< 1%) have been included (cf. Table IV).

Methylation analysis.—A slight modification of the method of Stevenson and Furneaux[10] (Hakomori technique on the triethylammonium salt of the carrageenans) was used to methylate the carrageenans. However, under the conditions used (the sodium salt of the methylsulfinyl carbanion instead of the potassium salt[10] and a higher polysaccharide to carbanion ratio), 10 min stirring[10] with the methylsulfinyl carbanion was insufficient to methylate λ-like carrageenans. This may be due to the decreased reactivity of the sodium salt of the anion[11]. Carrageenans C_1 and T were methylated employing different periods of reaction with the carbanion (Table III). It is evident that for κ/μ-carrageenans 10 min is enough to fully methylate the sample. On the other hand, at 10 min, λ-carrageenans showed significant undermethylation. After 100 min the methylation may be considered complete.

The seven fractions were subjected to methylation (Table IV). As expected, the cystocarpic fractions belong to the κ-family, with a κ/ι character in C_1, C_2, and C_3 and a μ/ν-character in C_4. The minor fractions C_2 and C_3 appear to be somehow richer in 3-linked disulfated (mostly on 2,6-) units than the major fractions C_1 and C_4 (Table IV). The two intermediate tetrasporic fractions, T_1 and T_2, are from the classical λ-family, though no evidence of 3-linked nonsulfated units were found (Table IV). The soluble tetrasporic fraction T_3 is very unusual: besides the presence of L-galactose, there are significant amounts of xylose and galactose branches (Table IV). The positions of sulfate groups, linkages, and branching in this fraction are unknown, as the pattern is different from that of a carrageenan.

TABLE IV

Molar ratio of methylated galactoses after permethylation and hydrolysis of the fractions obtained from cystocarpic and tetrasporic plants from *Iridaea undulosa*

Methyl group on	C_1	C_2	C_3	C_4	T_1	T_2	T_3 [a]
2,3,4,6-Tetra	Tr. [b]			Tr.		Tr.	13.9
2,4,6-Tri	Tr.	Tr.	Tr.	1.7	1.1	1.0	9.5
2,3,6-Tri	Tr.	Tr.	Tr.	1.3	Tr.	Tr.	6.5
2,3,4-Tri	Tr.						
3,4,6-Tri				Tr.	Tr.	Tr.	1.6
2,6-Di	91.5	88.5	88.8	82.7	1.2	2.2	16.2
4,6-Di	↑ 2.5			1.2	66.2	47.1	10.6
3,6-Di	↓	Tr.			Tr.	2.5	
2,3-Di	2.1	1.9	2.5	3.5	1.4	2.2	4.3
2,4-Di	Tr.	Tr.		1.2	Tr.	Tr.	25.2
6-Mono	Tr.	2.8	1.3	1.6	4.8	8.6	8.0
2-Mono	1.3	1.0	Tr.			Tr.	1.7
3-Mono	2.6	2.0	4.9	6.7	21.1	25.2	Tr.
4-Mono		3.8	2.4		4.1	3.4	Tr.
Gal					Tr.	7.6	

[a] Besides, 2.5% of 2,3,4-tri-*O*-methylxylose was found. [b] Tr., traces (< 1%).

The presence of 2,3-di- and 3-*O*-methylgalactose in the permethylated carrageenans does not correspond to the amount expected (Table II), due to the fact[3] that 4-linked 6-sulfated units are cyclized to 3,6-anhydro units in the alkaline methylation environment. This reaction occurs to an extent of 75–85% for the cystocarpic samples, to 100% for T_3, and to 50% for the tetrasporic fractions, showing that the cyclization reaction is slower in λ-carrageenans[12].

Table V shows the composition of the carrageenans in structural units. Table VI shows the correlation between the content of 3,6-anhydrogalactose, total sulfate,

TABLE V

Composition in structural units of cystocarpic and tetrasporic [a] carrageenans from *Iridaea undulosa*

	Percentage of sugars carrying sulfate at the indicated position [b]											
	3-Linked Gal							4-Linked Gal				
	–	2	4	2,4	2,6	4,6	Tri	–	2	6	2,6	3,6-AnGal +2-sulfate
C_1		1	43			1				5	12	38
C_2			43	1	2	1				11	5	36
C_3			46	1	1					2	16	34
C_4	1		33	1					1	12	20	32
T_1	1	47	1	3	3					2	43	
T_2	1	31	1	6	2		5		2	2	50	1

[a] The fraction T_3 is not included due to the presence of a non-carrageenan structural pattern. [b] Values are corrected considering the amounts of 3,6-anhydro-D-galactose present and the cyclization of 6-sulfated units in the methylation medium.

TABLE VI

Correlation of analytical factor and solubility of carrageenans isolated from cystocarpic samples from *Iridaea undulosa* and *Gigartina skottsbergii* [a]

Fraction	Solubility (M KCl)	3,6-AnGal	Sulfate	Gal 6-S	Gal 2,6-S	Gal 6-S total [b]
		per 100 sugars				
$1C_1$ *G.sk.*	0.30–0.31	39.0	80.6		4.3	4.3
$1C_2$ *G.sk.*	0.40–0.42	38.3	74.3	3.0	5.6	8.6
C_1 *I.und.*	0.50–0.70	38.0	61.2	4.8	11.9	16.7
C_2 *I.und.*	0.90–1.00	36.1	66.2	11.3	4.6	15.9
C_3 *I.und.*	1.30–1.40	34.3	63.7		18.2	18.2
C_4 *I.und.*	soluble	31.7	75.4	11.5	20.1	31.6
$1C_3$ *G.sk.*	soluble	26.9	83.8	7.2	21.5	28.7

[a] From this work and ref. 7. [b] Sum of Gal 6-sulfate plus Gal 2,6-disulfate (per 100 sugars).

α-D-galactose 6-sulfate, and α-D-galactose 2,6-disulfate with solubility for the cystocarpic stages from *Gigartina skottsbergii*[7] and *Iridaea undulosa*.

DISCUSSION

This study of the properties and structures of all the carrageenans produced by the cystocarpic and tetrasporic stages from *Iridaea undulosa*, and their correlation with previous data[1–4,7–9], allows us to investigate some of the problems in the chemistry of these polysaccharides: (a) the formal pattern of the systems biosynthesized by each stage of the seaweed and the relationships between the components, (b) general relationships between primary structure and solubility in potassium chloride solutions, and (c) the solubility of carrageenan mixtures versus that of "pure" compounds in these solutions.

Cystocarpic stage.—This stage from *Iridaea undulosa* produces, possibly from a common, heterodisperse μ/ν precursor, a system of carrageenans composed by two main fractions, in similar amounts (Table II); namely, a KCl-soluble, partly cyclized μ/ν-carrageenan and a gel-forming κ/ι-carrageenan which precipitates at "intermediate" concentrations of KCl. Small amounts of two other carrageenans with analytical characteristics intermediate between the major members of the family, but with gel-forming properties, optical rotations, and methylation analysis similar to the κ/ι-fraction were also detected. The system is similar, from a formal point of view to that produced by the same stage of *Gigartina skottsbergii*[7]. It is noteworthy to find κ/ι-carrageenans with these high solubilities in KCl solutions.

The significant increase in optical rotation when passing from the μ/ν-fraction to the precipitable carrageenans may be due either to a conformational change to a more organized state, or to the presence in the soluble fraction of some impurities that give rise to low optical rotation. The last hypothesis is supported by the fact that such a change was not found in the study of the cystocarpic system from *Gigartina skottsbergii*[7]. The data in Table II also suggest that if the three κ/ι products (C_1, C_2, and C_3) are biosynthesized from the partly cyclized μ/ν-carra-

geenan C_4, there is not a single cyclization sequence ($C_4 \rightarrow C_3 \rightarrow C_2 \rightarrow C_1$). On the other hand, for the *Gigartina skottsbergii* carrageenans, the production of $1C_1$ and $1C_2$ from $1C_3$ is consistent with the single cyclization sequence[7].

A sharp decrease in the amounts of cyclizable 6-sulfated plus 2,6-disulfated α-D-galactosyl units occurs when passing from the μ/ν-carrageenan (C_4, 31 units per 100 sugar residues) to the three κ/ι-fractions (16–18 units per 100 sugar residues), as happened with the *Gigartina skottsbergii* carrageenans[7] (Table VI). It has been considered for a long time that the content of 3,6-AnGal and that of sulfate are inversely and directly proportional to the KCl solubility of carrageenans[13], respectively. Table VI shows (for carrageenans from *Iridaea undulosa* and *Gigartina skottsbergii*) agreement with this with regard to the 3,6-AnGal content. Also a correlation of solubility with 6 sulfated α-galactose units (Gal 6-S + Gal 2,6-di-S) is shown: the carrageenan with the lower proportion of 6-sulfated α-D-galactose units has the lower solubility in KCl solutions. However, the percentage of total sulfate can be the same in μ/ν- and κ/ι-carrageenans with different solubility, and no correlation was found between 6-sulfated or 2,6-disulfated units and solubility (Table VI).

Tetrasporic stage.—This stage of *Iridaea undulosa* biosynthesizes λ-carrageenans with 90% yield. Its arbitrary fractionation shows a common basic structure with higher sulfate content in the more soluble fraction T_2. In this carrageenan, an average of 37% of the units carry two sulfate moieties; possibly the ionization of the neighboring hydroxyl groups during the methylation is shielded, thus restricting the permethylation of the carrageenan (Table IV). The fraction soluble in potassium chloride (T_3) appears to be a mixture of at least two different polysaccharides. One of them, > 60%, contains L-galactose, and thus is not a carrageenan. The presence of L-galactose as a component of the polysaccharides biosynthesized by carrageenophytes[14] suggests that the enzyme system of these seaweeds is much more complicated than expected.

The system of carrageenans from the tetrasporic stage from *Iridaea undulosa* is similar to that from the same stage of *Gigartina skottsbergii*[7]. The systems of carrageenans synthesized[5,15] by gametophytes and tetrasporophytes of *Chondrus crispus* are also similar to those mentioned above.

Solubility of "pure" carrageenans from separated life phases versus mixtures of carrageenans extracted from unsorted samples.—The only procedure for separating a partially cyclized μ/ν- and a λ-carrageenan from unsorted sexual stages of the seaweed was through the alkali-treated derivative, by precipitation with potassium chloride. On the other hand, the routine use of 0.4 M KCl solutions for the separation of gelling (κ-) and "soluble" carrageenans has precluded the investigation of their solubilities at higher concentrations of the salt. Partly cyclized μ/ν-carrageenans are now easily isolated from cystocarpic samples, and "pure" λ-carrageenans from tetrasporic ones, leading one to appreciate the fact that the former are completely soluble in KCl solutions, while the latter actually precipitate at high concentrations of this salt.

Fig. 1 depicts the yields, precipitation ranges, and analysis of the fractions obtained from separated cystocarpic and tetrasporic plants, compared with those obtained from unsorted samples of the seaweed. From the amount of κ/ι-like material in the carrageenan from the unsorted sample, it seems to be composed of a mixture of about two thirds of cystocarpic carrageenans and one third of tetrasporic carrageenan. However, the carrageenans precipitate at higher KCl concentrations, as expected for mixtures: 0.70–1.05 M for κ/ι-samples vs. 0.50–0.70 M for isolated cystocarpic ones, and 1.20–1.65 M vs. 1.00–1.20 M for the tetrasporic samples. Another concern, is that the KCl-soluble material in the mixture is much less than that accounted for in the sorted samples.

These data override a previous suggestion[13] that the solubility of carrageenan molecules in mixtures was the same as that in homogeneous fractions, i.e., that the KCl precipitation range should define the chemical structure of the carrageenan. This is not the case, even for "pure" compounds, e.g., κ/ι-carrageenans C_2 and C_3 precipitate at KCl ranges similar to the λ-carrageenan T_1. It should be emphasized the importance of using sorted sexual individuals of the seaweed as starting materials, thus producing the simplest systems attainable at this moment.

Given these facts, it is not longer valid to speak of a "carrageenan family". The κ-family[16], produced by cystocarpic samples, and the λ-family[16], produced by tetrasporic ones, should be considered as different polysaccharide families, just as agar and carrageenans are considered today.

EXPERIMENTAL

Samples of *Iridaea undulosa* were collected near Puerto Madryn (Province of Chubut) and sorted at the Centro Nacional Patagónico (Puerto Madryn, Chubut).

Extraction and fractionation.—The cystocarpic and tetrasporic plants were milled and extracted with water (20 g/L) at room temperature, with mechanical stirring for 16 h. The residues were removed by centrifugation, and the supernatants were poured into 3 vol of 2-propanol. The precipitated polysaccharides (CWS-1 and TWS-1) were dried by solvent exchange and finally in vacuo. The residues were extracted two additional times in the same fashion. The alcoholic solutions used for the precipitation rendered, after settling, fine precipitates of carrageenans (CWS-F and TWS-F), which were isolated by centrifugation and solvent exchange. The final residues were extracted with boiling water, for 6 h, yielding, by the above-mentioned procedure, carrageenans CHS and THS. The products extracted at room temperature were pooled by dissolution, centrifugation, and lyophilization of the supernatant, yielding carrageenans C and T, respectively.

For fractionation, the carrageenan was dissolved in water (2.5 g/L), and solid ground KCl was added in small portions, with violent stirring, so that the concentration was increased by 0.1–0.2 M each time. After each addition the stirring was continued for 6 h to ensure equilibration, and the solutions were centrifuged. The

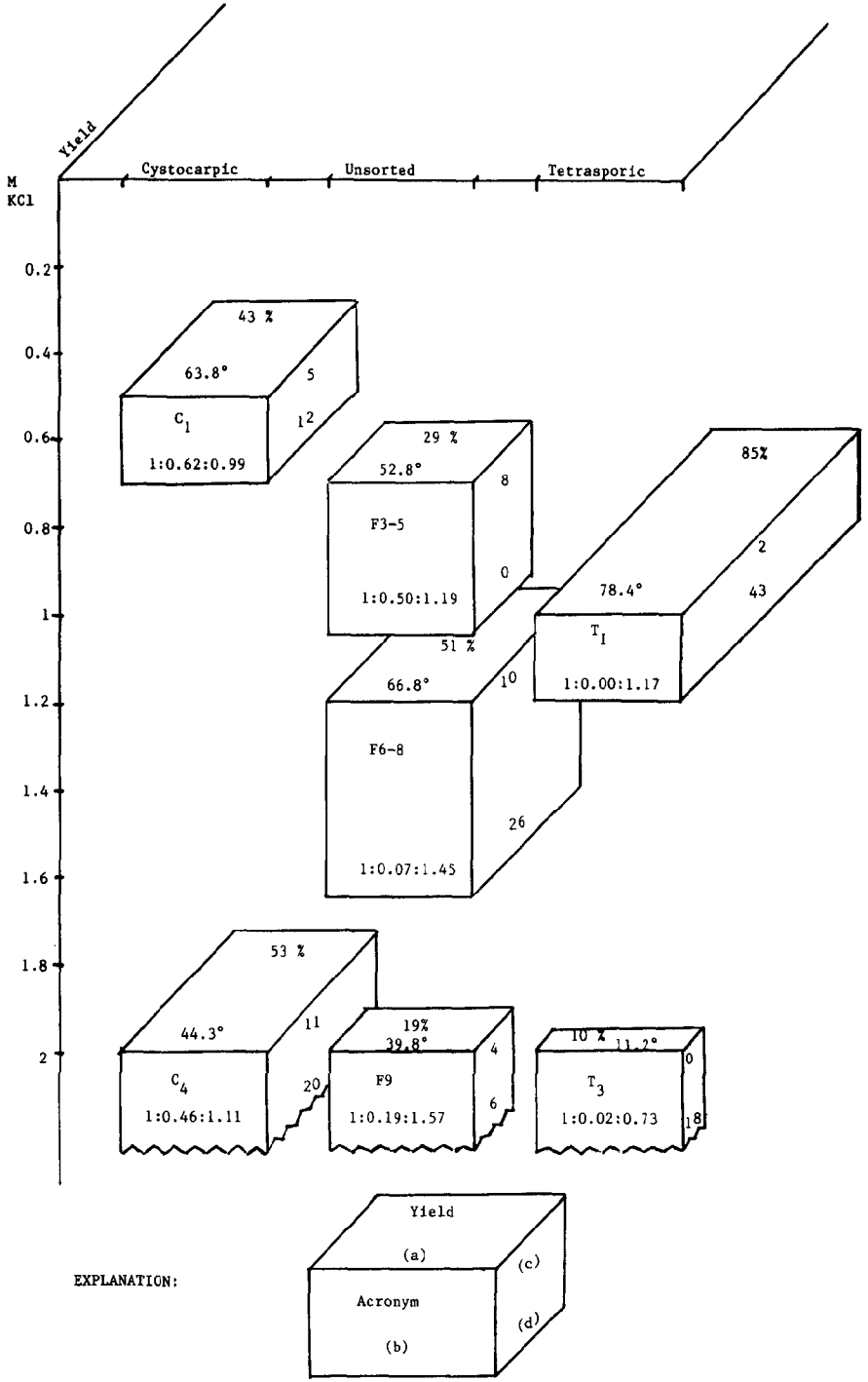

Fig. 1. Yields and analysis of the main fractions isolated from cystocarpic, tetrasporic and unsorted samples from *Iridaea undulosa*. Key: (a) $[\alpha]_D$ (°); (b) Molar ratio Gal:3,6-AnGal:sulfate; (c) Gal 6-sulfate/100 sugars; and (d) Gal 2,6-disulfate/100 sugars.

precipitates, as well as the residual solution (upper limit 2 M KCl), were dialyzed, concentrated, and lyophilized.

Methylation analysis.—The polysaccharides were converted to their triethylammonium salts[10] by ion-exchange with Dowex-50 resin, previously treated with $Et_3N \cdot HCl$, and washed with water. After lyophilization and extensive drying, polysaccharides (5 mg) were dissolved in Me_2SO (0.7 mL) with stirring. Sodium methylsulfinylmethanide (2 M in Me_2SO, 0.7 mL) was added to each vial, with stirring for 40 min (cystocarpic samples) or 100 min (tetrasporic samples). Methyl iodide (0.6 mL) was added under an ice bath, and reaction was allowed to proceed at room temperature for 0.5–1 h. The mixture was mixed with water and dialyzed exhaustively against tap water, then against distilled water, followed by lyophilization. Methylated polysaccharides were hydrolyzed with 45% formic acid for 16 h at 100°C. After evaporation of the acid, and addition of dil NH_3 solution, the residue was extracted with abs EtOH. The solvent was evaporated off and the residue converted to the acetylated aldononitriles[17], which were analyzed by gas–liquid chromatography on a fused silica column coated with SP-2330 (30 m × 0.25 mm; film thickness, 0.20 μm), running isothermally at 210°C (FID and injector temperature, 230°C). Some results were confirmed by the use of alditol acetates, run under the same conditions.

General and analytical methods.—Galactose was determined by the method of Dubois et al.[18] and corrected for the presence of 3,6-anhydro-D-galactose, which was independently determined by the resorcinol–HCl method[19]. Sulfate was determined by the method of Dodgson and Price[20], and total 6-sulfate by 3,6-anhydro-D-galactose measurement[19] after alkaline treatment (1 M NaOH, 80°C, 2–16 h). The 2,6-disulfate was determined by performing the previously mentioned procedure to a product previously treated with $NaIO_4$ (0.015 M, overnight, room temperature). Optical rotations were measured using 0.4–0.6% solutions in 0.1 M NaCl. The solutions were equilibrated in the polarimeter tube overnight before the determinations were made. The configuration of the galactose present was determined by separation and quantitation of their acetylated (−)-2-octylglycosides[21] by gas–liquid chromatography on a fused silica column coated with HP-5 (50 m × 0.32 mm; film thickness, 0.17 μm), running isothermally at 210°C (FID and injector temperature, 240°C).

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