"A STRUCTURAL INVESTIGATION OF THE SULPHATED POLYSACCHARIDE FROM *Aeodes ulvoidea* Schmitz"

by

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A Thesis

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CONTENTS

SUMMARY

Aeodes ulvoidea, a red seaweed of the *Grateloupiaceae*, yielded a highly sulphated polysaccharide which was shown to contain D- and L-galactose, 4-O-methy-L-galactose, 2-O-methyl-D- and L-galactose and 6-O-methyl-D-galactose, together with chromatographic traces of xylose and mannose. The sulphate was not labile to alkali, but it was largely removed with methanolic hydrogen chloride. Periodate oxidation of the polysaccharide, methylation of the desulphated polysaccharide, and investigation of fifteen oligosaccharides from partial hydrolysis and acetolysis studies of the polysaccharide, indicate that (a) the polysaccharide is composed of a backbone of D-galactose residues which are 1,3- and 1,4-linked (b) at least some regions of alternating structure do occur (c) the 2-O-methylgalactose is linked through the 4-position (d) the 4-O-methyl-L-galactose is present as single unit side chains glycosidically linked to the galactose backbone at position 6, and (e) most of the 6-O-methyl-D-galactose is linked to the 4-position of 2-O-methyl-D-galactose.

1. INTRODUCTION.

POLYSACCHARIDE SULPHATES OF THE RHODOPHYCEAE

Most marine algae thus far examined contain sulphated polysaccharides, a feature absent in land plants. The biological functions of the carbohydrate sulphates, unlike the carbohydrate phosphates, appear to be connected more with their physical properties than with their chemical reactions [1]. In the Rhodophyceae the sulphate hemi-ester groups are attached to galactans, while in the Chlorophyceae and the Phaeophyceae they are attached mainly to arabinogalactans and fucoidan respectively.

The red seaweed galactans are mucilaginous in nature and occur both in the cell wall and intercellularly. Most of these polysaccharides consist of galactose or modified galactose units linked α-1,3 or β-1,4 in alternating sequence [2]. These units may take the form of D- or L-galactose, 3,6-anhydro-D- or L-galactose, 3,6-anhydro-2-O-methyl-L-galactose and 2-, 3-, 4- and 6-O-methylgalactoses, with further masking of some of these units with hemi-ester sulphate and pyruvic acid groups. "It would appear that the sulphated galactans of the red seaweeds are a family of related molecules, each with properties appropriate for a particular species growing in a particular environment" [2]. In the discussion which follows these galactans will be treated under four main headings *viz*: Agar-type, Carrageenan-type, Grateloupiaceae and Miscellaneous polysaccharides.

1.1. Agar-type polysaccharides

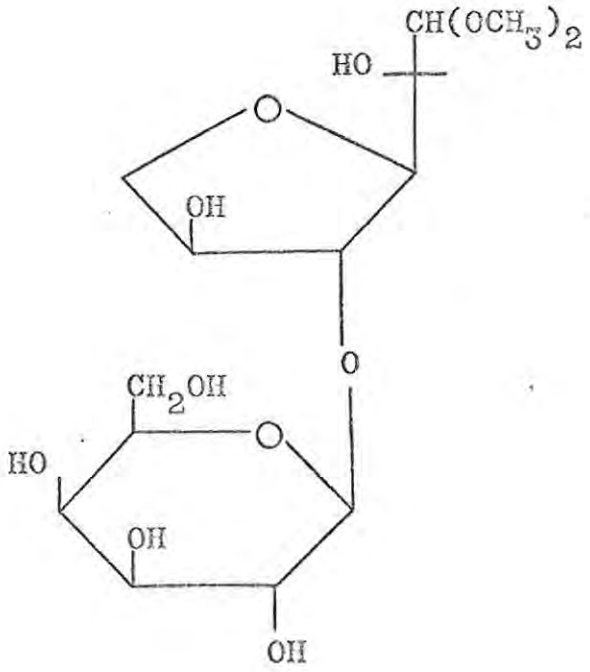
1.1.1. Agar

Agar is the polysaccharide complex synthesised by species of Gelidium, Gracilaria, Acanthopeltis, Ahnfeltia, Ceramium, Campylaephora, Phyllophora and Pterocladia species; the chief sources are Gelidium species and the largest producer is Japan where it has been available commercially since the seventeenth century. Agar is extracted by boiling the seaweed in water and allowing the solution to set to a gel. Commercial use of agar is confined mainly to its gelling properties, the strength of the gel varying with the source of the agar [3]. The gelling properties are due to the virtually neutral polysaccharide complex, agarose, which constitutes the major "component" (but see later) of commercial agar. Agar was first fractionated by Araki [4] by chloroform extraction of its acetate, the chloroform-soluble fraction being termed agarose acetate (70%) and the insoluble fraction agaropectin acetate (30%). Other methods of separation have been developed, since agarose is a more desirable matrix for gel-electrophoresis [5] or gel-chromatography than the unfractionated agar. These include precipitation of the agaropectin with cetylpyridinium chloride [6] or with chitosan [7], and precipitation of agarose from a hot agar solution with polyethylene glycol 6000 [8].

Early investigations on unfractionated agar established D-galactose as the predominant component [9,10]; the chief hydrolysis product of methylated agar was 2,4,6-tri-O-methyl-D-galactose [11,12] establishing the linkage through the 3-position. No 3,6-anhydrogalactose was reported in these hydrolysis studies since it is very unstable to acid hydrolysis,

being decomposed to 5-hydroxy-2-furfuraldehyde which is further degraded to levulinic acid and formic acid [13]. Thus it was thought at the time that the main structural feature of agar was a chain of β-D-galactopyranosyl units linked 1→3 [14]: the small amount of 3,6-anhydro-L-galactose isolated from methylated agar was thought by some workers [15] to be an artefact produced from 1,4-linked L-galactose 6-sulphate units by desulphation [14] under the alkaline conditions of methylation. This view was rejected by others [16] since the sulphate content of native agar is too low to account for the yields of 3,6-anhydro-L-galactose isolated: the real presence of 3,6-anhydro-L-galactose in native agar was later firmly established [16,17,18,19]. The mode of linkage was elucidated by the isolation of 2-O-methyl-3,6-anhydro-L-galactose dimethyl acetal from the methanolysate of methylated agar [18] (from G. amansii). This was confirmed by the isolation and characterization of agarobiose [19] from a partial hydrolysis of agar, and by the isolation of agarobiose dimethyl acetal [20] I in 70% yield from a partial methanolysis of agar.

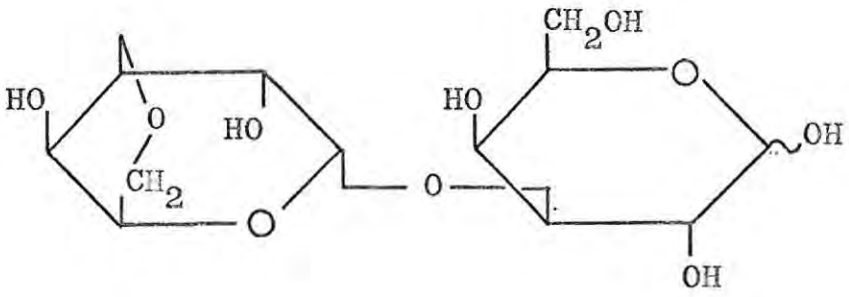
I



The isolation of agarobiose proves that the D-galactopyranosyl residues are joined β-1,4 to the 3-6-anhydro-L-galactose residues in the macromolecule, while the high yield indicates that the disaccharide units constitute most of the macromolecule.

The final link in the elucidation of the gross structure of agar came with the isolation of the crystalline disaccharide neoagarobiose [21] II and the crystalline tetrasaccharide neoagarotetraose [22] III ; both were derived from an enzymic hydrolysis of agar by Pseudomonas kyotoensis.

II



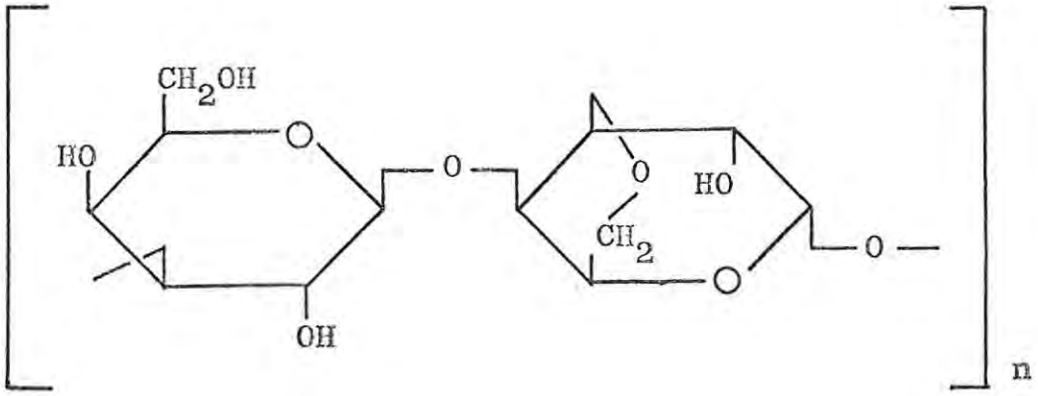
III $\alpha\text{-}A_L(1\rightarrow 3)\text{-}\beta\text{-}G_D(1\rightarrow 4)\text{-}\alpha\text{-}A_L(1\rightarrow 3)\text{-}\beta\text{-}G_D$

where A_L = 3,6-anhydro-L-galactose

G_D = D-galactose

Thus in 1956 Araki [23] proposed structure IV for the agarose "fraction" of G. amansii. A similar structure was suggested by O'Neill and Stewart [24] for the unfractionated agar of Gelidium cartilagineum (but with hemi-ester sulphate on every tenth galactose residue) and by Clingman et al [25] for the unfractionated agar of Gracilaria confervoides (with hemi-ester sulphate on every fortieth galactose residue), indicating that the polysaccharides from various agarophytes and from very different locales can have similar structures.

IV

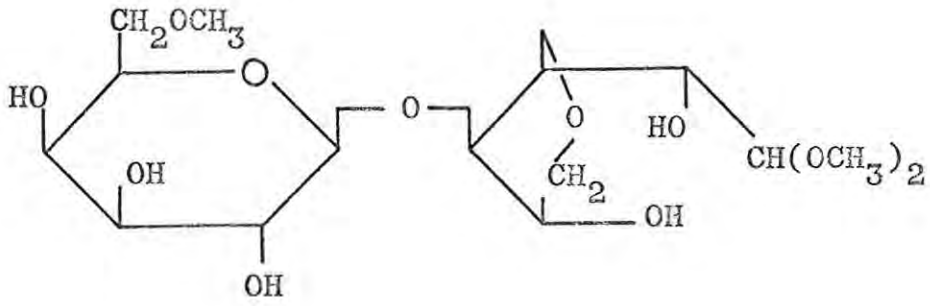


In addition to D-galactose and 3,6-anhydro-L-galactose, the following neutral saccharides have also been reported to be present to a varying extent in agar: L-galactose, D-xylose, 4-O-methyl-L-galactose and 6-O-methyl-D-galactose. The yield of 4-O-methyl-L-galactose from G. amansii [26] was so low that it does not seem to have any structural significance. The mode of linkage of this sugar in the polysaccharide is not yet reported, but since it is methylated in the 4-position, it is obvious that this L-galactose-derived residue is not 1,4-linked in the macromolecule.

6-O-Methyl-D-galactose, which has long been known to be a constituent of porphyran [27], was found to be present in eight agarophytes investigated by Japanese workers [28]. The mode of linkage was elucidated by the isolation of the disaccharide dimethyl acetal V from Ceramium boydenii (which has a very high 6-O-methyl-D-galactose content). This proves that the 6-O-methyl-D-galactose residues are linked in a similar manner to the D-galactose residues. Furthermore, although the amounts of D-galactose and 6-O-methyl-D-galactose vary widely among the various "agaroses", the total amount of these two saccharides

always constitutes 51 - 53% of the polysaccharide.

V



This suggests that some of the D-galactose residues in the repeating sequence are merely replaced by the corresponding 6-O-methyl ether. Also, the 3,6-anhydro-L-galactose content is always 44 - 45% for any agarose, and the sum of the molar ratios of these three sugars amounts to more than 95% of the polysaccharide molecule. Finally, the L-galactose and D-xylose content of the polysaccharides is so low that Araki regards them as contaminants [28]. Investigation of the agars from six Gracilaria species [29] viz. G. debilis, G. compressa, G. foliifera, G. domingenis, G. damaecornis and G. ferox gave similar results and a number of common trends in their composition were observed. Thus the molar ratio of D-galactose plus 6-O-methyl-D-galactose to 3,6-anhydro-L-galactose plus sulphate was approximately 1:1 for all of the polysaccharides, although the 6-O-methyl-D-galactose content ranged from 2.3 - 21%.

From all this data the general formula VI emerges for the neutral polysaccharide fraction of agar (after Araki [28]). The amount of 6-O-methyl-D-galactose varies widely from one agarophyte species to another.

$$A_L(1\rightarrow[3)\text{-}G_D(1\rightarrow 4)\text{-}A_L(1]_x\rightarrow[3)\text{-}G_D(1\rightarrow 4)\text{-}A_L(1]_y\rightarrow 3)\text{-}G_D$$

$$\text{6OMe}$$

VI : G_D = D-galactose

A_L = 3,6-anhydro-L-galactose

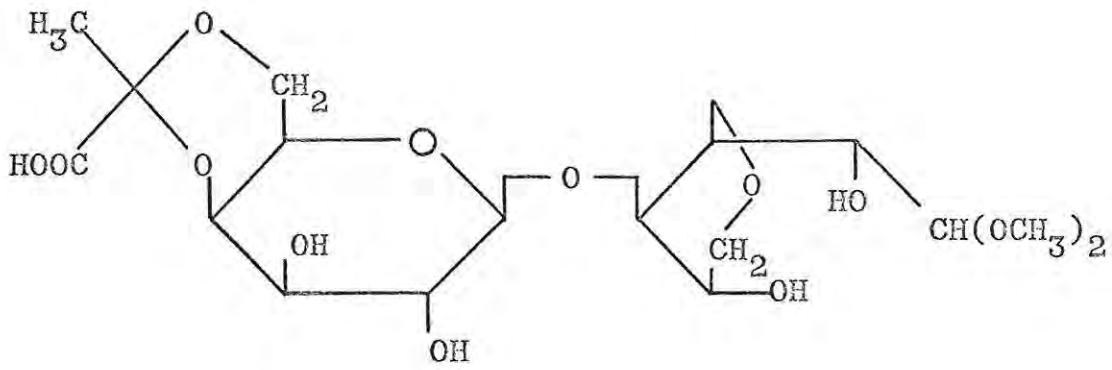
Thus in G. amansii the D-galactose : 6-O-methyl-D-galactose ratio is approximately 50 : 1 i.e. x = 50 and y = 1. Similarly for C. boydenii x = 3 and y = 2. Since neither D-galactose nor 3,6-anhydro-L-galactose was detected in the enzymic hydrolysates of agar (which involved the cleavage of β-glycosidic linkages), it was concluded that the polysaccharide chain is terminated on the non-reducing end by 3,6-anhydro-L-galactose and on the reducing end by D-galactose. This is, of course, purely a hypothetical formula since the relative positions of D-galactose and 6-O-methyl-D-galactose in the macromolecule are unknown.

A further way in which the D-galactose residues can be masked is by the presence of pyruvic acid : the mode of linkage of this acid was elucidated [30] by the identification of the disaccharide VII, 4,6-O-1'-carboxyethylidene-β-D-galactopyranosyl-(1→4)-3,6-anhydro-L-galactose dimethyl acetal from a partial methanolysate of agar. Araki [28] has shown that the agars of G. amansii and G. subcostantum contain about 1% pyruvic acid.

The pyruvic acid content of the six Gracilaria species polysaccharides [29] mentioned above varied from 0.1 - 2.9%. the highest pyruvic acid content occurring when the sulphate content was above 4% and the 6-O-methyl-D-galactose content was low, while low pyruvic acid content was favoured by high 6-O-methyl-D-galactose content. This is explained if the

4,6-acetal is added during biogenesis at the polysaccharide level since the number of D-galactose residues available for formation of this 4,6-acetal depends on the extent of 6-O-methylation and on sulphation.

VII



Although discussion thus far has been centered for convenience around the structure of the agarose "component" of agar, present indications [3,31,32,33] are that agar is composed of a whole spectrum of polysaccharides and not simply the agarose and agaropectin fractions mentioned above. Thus Duckworth and Yaphe [31] fractionated Difco Bacto agar (probably from G. cartilagineum) by using the charge-dependant solublility of the polysaccharides in water at various temperatures : by washing the agar under varying conditions they obtained fractions which varied widely in 3,6-anhydro-L-galactose, sulphate and pyruvate content. They achieved a more complete fractionation of the commercial agar on a DEAE Sephadex A-50 (Cl^-) column by stepwise elution with NaCl solution (Table I ; after Duckworth and Yaphe [31]). These results have been interpreted as follows :

(i) the low total polysaccharide recovery is due to highly charged galactans not removed in the freezing and thawing during

initial purification of the agar, and which are irreversibly bound to the column.

TABLE I : FRACTIONATION OF DIFCO BACTO AGAR BY COLUMN CHROMATOGRAPHY ON DEAE SEPHADEX A-50 (AFTER DUCKWORTH AND YAPHE [3]).

Polysaccharide fraction	Eluant	Yield[a] (%)	3,6-anhydro-L-galactose (%)	Sulphate (%)	Pyruvic acid (%)	Gel formed[b]
a	distilled water	25.5	47.8	0.08	0.02	++++
b	0.2M NaCl	1.9	44.1	0.94	0.06	+++
c	0.5M NaCl	25.5	44.0	1.07	0.09	++
d	0.8M NaCl	3.8	19.3	3.40	0.97	-
e	1.2M NaCl	1.9	12.0	5.80	0.64	-
f	1.8M NaCl	0.3	9.9	6.00	0.70	-
g	2.4M NaCl	0.3	9.0	6.00	0.50	-
h	3.0M NaCl	0.3	5.0	7.00	0.50	-
		59.5				

(a) Total yield, 59.5% of the polysaccharide added to the column; (b) + is indicative of gel strength of a 1% solution, - no gel at a concentration of 1%.

(ii) Although, as would be expected, the sulphate content of the polysaccharides increases with the ionic strength of the eluant, nevertheless the pyruvate content reaches a maximum at 0.8M NaCl. This indicates that the masking of the D-galactose residues with 4,6-carboxyethylidene-D-galactose residues occurs away from the sulphated galactose residues (cf. the six Gracilaria species agars mentioned above).

(iii) The gel strength decreases with the increasing degree of sulphation and the decreasing 3,6-anhydro-L-galactose content. These observations are in agreement with Rees'

concept [34] that the gel-forming capability is due to the three equatorial hydrogen atoms on the 3,6-anhydro-L-galactose residues which constrain the polysaccharide into a helix. Interaction of helices causes gel formation. Any replacement of a 3,6-anhydro-L-galactose residue by a sulphated galactose residue changes the conformation of the L-residue, thereby causing a kink in the helix, and impairing the gelling ability. It is of interest to note that the gel strength is not dependant on the 6-O-methyl-D-galactose content. However, the ease with which the gel forms, i.e. the temperature at which it forms, is directly proportional to the methoxyl content [35].

The third fractionation method used by Duckworth and Yaphe [31] relies on the fact that at low pH the "sulphated" molecules are still fully ionized while the net charge on the "pyruvated" molecules is considerably reduced. Thus a pyruvate-enriched sample, collected by washing commercial agar with water at 70°, was further fractionated on DEAE Sephadex A-50 at pH 3.4 by gradient elution with buffered NaCl solution. Although complete separation was not achieved, pyruvate-enriched and sulphate-enriched fractions were obtained. On the basis of the above results these workers have concluded that agar consists of a whole spectrum of polysaccharides varying in both the quantity and nature of the charged groups on the macromolecule. From this study it is concluded that there are three extremes of structure for whole agar :

(a) Neutral agarose i.e. a chain of alternating 1,4-linked 3,6-anhydro-L-galactose and 1,3-linked D-galactose residues containing no charged groups.

(b) Pyruvated agarose with little sulphation. Here some of the D-galactose residues are masked by substitution as 4,6-carboxyethylidene-D-galactose, the degree of substitution increasing to about one in twenty, at which point the degree of sulphation is about 2% (replacing some of the 3,6-anhydro-L-galactose residues with galactose sulphate).

(c) Sulphated galactan containing no or little 3,6-anhydro-L-galactose or 4,6-carboxyethylidene-D-galactose residues.

From the extreme case (b) the concentration of pyruvate decreases as the sulphate content increases until the extreme of a non-gelling sulphated galactan is reached. The ratio of these three extremes of structure are represented in Figure I ;

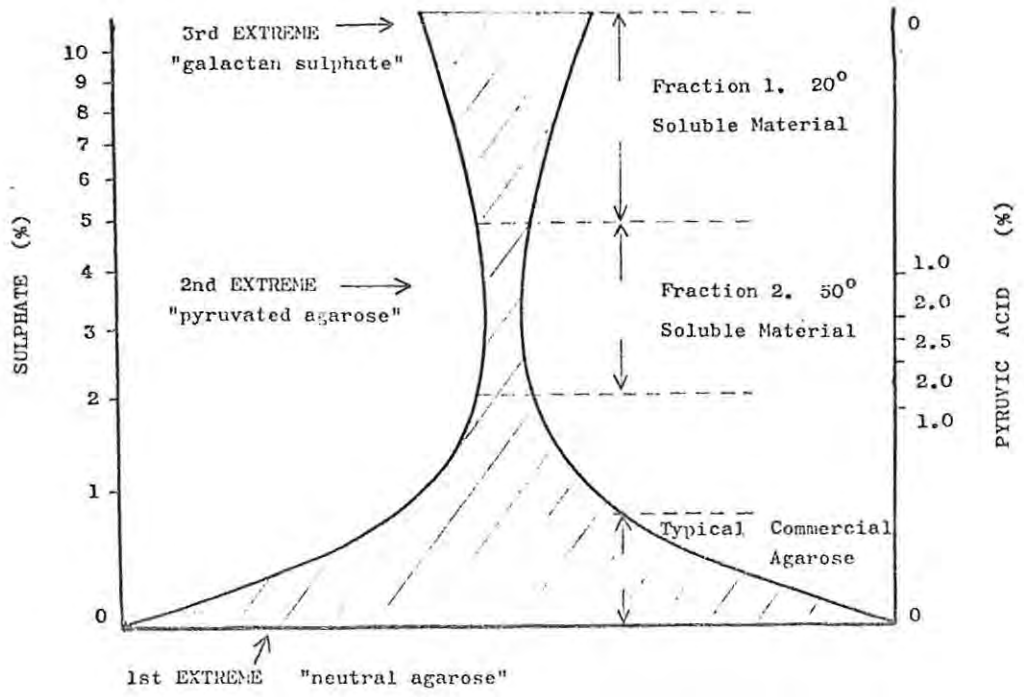


FIGURE I : (After Duckworth and Yaphe [31]). The complexity of the spectrum of polysaccharides in Difco Bacto agar. The total shaded area represents complete agar. The area within the curves between any lines drawn horizontally indicates the approximate amount of polysaccharide having that number of charged groups.

The presence of a few charged groups in commercial agarose has in the past been generally accepted as being due to inefficient fractionation of the neutral molecules from charged agaropectin, but it is now clear that agarose is merely a mixture of these molecules having the lowest charge content and the greatest gelling ability.

In order to elucidate further the fine structure of agar, Duckworth and Yaphe [32] investigated three agar fractions corresponding as closely as possible to the idealised extremes of structure ((a),(b) and (c)) mentioned above. These fractions were incubated with an extracellular agarose from Pseudomonas atlantica. As the sulphate content of these fractions increased so did the proportion of polysaccharide remaining after enzyme action, indicating that the enzyme cannot cleave those portions of the macromolecule rich in sulphate. Fraction (a) yielded, as expected, a mixture of mainly neutral oligosaccharides viz. neoagarohexaose (A_L-G_D-A_L-G_D-A_L-G_D where G_D=D-galactose and A_L=3,6-anhydro-L-galactose), neoagarotetraose III and neoagarobiose II in the ratio 6 : 7 : 1. Fractions (b) and (c) yielded a complicated mixture of neutral and charged oligosaccharides which were further separated on DEAE Sephadex A-25 (Cl^-). The neutral oligosaccharides from both these fractions were the same as those from the agarose fraction (a), indicating that the uncharged portions of the extreme molecule structures (pyruvated agarose and galactan sulphate) have regions which approximate to agarose. The charged oligosaccharides from the pyruvated agarose (b) consist mainly of a hexasaccharide VIII and a tetrasaccharide IX.

VIII A_L-G_D-A_L-G_D-A_L-G_D
|
4,6-carboxyethylidene

IX A_L-G_D-A_L-G_D
|
4,6-carboxyethylidene

G_D= D-galactose
A_L= 3,6-anhydro-L-galactose

The large yield of these two charged oligosaccharides free of sulphate is yet again indicative of the occurrence of 4,6-carboxyethylidene-D-galactose residues in regions of the macromolecule low in sulphate. Furthermore, the enzyme resistant portion of the polysaccharide was higher in sulphate and lower in pyruvate content than the original polysaccharide. The sulphate-rich charged oligosaccharides had a high molecular weight.

The charged oligosaccharides obtained from the sulphated galactan (c) were also separated on DEAE Sephadex G-25, and were found to contain sulphated and pyruvated oligosaccharides in the ratio 4 : 1, the two pyruvated oligosaccharides being identical with those from fraction (b). Again the sulphated oligosaccharides were of high molecular weight. Further information on the sulphated region of agar was not obtained.

In conclusion it may be said that the enzymic hydrolysis of this agar further indicates that agar consists of a spectrum of polysaccharides having the above three extremes of structure. Fractionation of several agars [33] rich in pyruvate indicates that it may be a general feature of agars that replacement of D-galactose with 4,6-carboxyethylidene-D-galactose occurs in those regions of the macromolecule low in sulphate.

Similar conclusions have been reached by Izumi [36] on the agar from G. amansii. The enzymic degradation of the six Gracilaria agars with P. atlantica gave four main neutral oligosaccharides which were shown to be 6^1-O-methylneoagarobiose, neoagarobiose, 6^3-O-methylneoagarotetraose and neoagarotetraose. Examination of the charged oligosaccharides confirms that Gracilaria agars consist of a closely related series of polysaccharides in which the extent of masking varies. In a further investigation on these Gracilaria agars, Duckworth et al [29] have made some interesting observations on the effect of the structure of agar on its gelling ability. The results are interpreted as follows.

(i) The only agar which has a high gel strength is that from G. debilis (pyruvate < 0.2%) and since a significant amount of the polysaccharide is eluted by water from a DEAE Sephadex A-50 column, they use this parameter (i.e. the percentage of the total polysaccharide eluted by water) as a measure of the gelling properties of the other unfractionated Gracilaria agars.

(ii) The agar from G. foliifera (pyruvate < 0.2%) has a good gel strength only after alkali treatment (sulphate content drops to ca. 1%) and a study of its elution pattern on DEAE Sephadex A-50 before and after alkali treatment indicates that it is the sulphate content and not the 6-O-methyl-D-galactose content which affects the gel strength of this agar.

(iii) The agar from G. compressa (pyruvate 2.9%) has a weak gel strength even after alkali treatment (when sulphate content drops to 1.3%). The bulk of this agar is eluted by 0.5M NaCl from a DEAE Sephadex A-50 column which is characteristic

of polysaccharides with a high 4,6-O-(1-carboxyethylidene)-D-galactose content. The weak gelling ability is thus caused by the majority of the polysaccharide molecules which approach the "pyruvated agarose" instead of the neutral agarose structure.

(iv) The agars of G. ferox, G. damaecornis and G. domingensis form the weakest gels of all. They have a high sulphate content, the resistance of which to alkali indicates that most of the sulphate is not located at C-6. The assumption that all the sulphate is attached to the L-galactose residues results in an anomalously high L- : D-galactose ratio for an agar, so that in fact some of the D-galactose residues could be sulphated. These agars are only partially eluted from DEAE Sephadex A-50 with 3M NaCl.

Thus it is clear that the gel strength of these polysaccharides decreases as the extent of masking of the basic agarose structure increases.

1.1.2. Porphyran

Several Porphyra species are used as food in various parts of the world (e.g. "laver bread" in Wales is made from P. umbilicalis). The porphyran-type of mucilage obtained from this genus is also found in species of Laurentia and in Bangia fuscopurpurea. Investigation of the mucilage from Porphyra capensis [37] showed D- and L-galactose, 6-O-methyl-D-galactose, 3,6-anhydro-L-galactose and sulphate in the ratio of 1 : 1 : 2 : 1. At first the presence of the 6-O-methyl-D-galactose was thought to distinguish this type of polysaccharide from agar. However, it now appears that porphyran resembles agar in containing 3,6-anhydro-L-galactose and 6-O-methyl-D-galactose and it resembles carrageenan in containing galactose

6-sulphate.

The porphyran obtained from P. umbilicalis was shown [38] to have a similar composition to that from P. capensis [37]. Careful degradation of the polysaccharide with dilute H_2SO_4 led to the isolation of L-galactose 6-sulphate [39] : but since the molar ratio of sulphate exceeds that of L-galactose, it must be assumed that other ester sulphates are also present. Rees [40,41] showed that an enzyme preparation from Porphyra extracts is able to convert the L-galactose 6-sulphate residues in the polysaccharide into 3,6-anhydro-L-galactose residues with concomitant release of the sulphate : this suggests that (i) the L-galactose 6-sulphate is the biological precursor of the 3,6-anhydro-L-galactose, and (ii) that since the 3,6-anhydro-L-galactose is usually combined in polysaccharides through the 4-position and with α-linkage, it is likely that the L-galactose 6-sulphate is similarly linked. Similar results were obtained by elimination of the sulphate with alkali [42].

Rees and Conway [43] showed that the composition of P. umbilicalis varies widely with the season and locality. However, although the molar percentage of 3,6-anhydro-L-galactose varies from 12 to 29, and that of L-galactose 6-sulphate from 17 to 37, nevertheless the sum of these two saccharides in any one sample varies only between 43 and 55%. A similar relationship exists between 6-O-methyl-D-galactose and D-galactose since the sum of their molar percentages constitutes 47 - 55% of the polysaccharide irrespective of the variation of the amount of the individual saccharides. These results are consistent with the view of Turvey and Williams [44] that the simplest structure of porphyran is a chain of alternating 1,3-β-linked D-galactose and 1,4-α-linked L-galactose units,

some of the D-galactose being 6-O-methylated and the L-galactose occuring either as the 6-sulphate or the 3,6-anhydride. In support of this view Turvey and Williams [44] have isolated the following disaccharides from a partial hydrolysis of porphyran :

X β-G_D(1→4)-G_L6-SO_4

XI 6-O-Me-β-G_D(1→4)-G_L6-SO_4

XII α-G_L6-SO_4(1→3)-G_D

XIII α-G_L6-SO_4(1→3)-6-O-Me-G_D

G_D= D-galactose

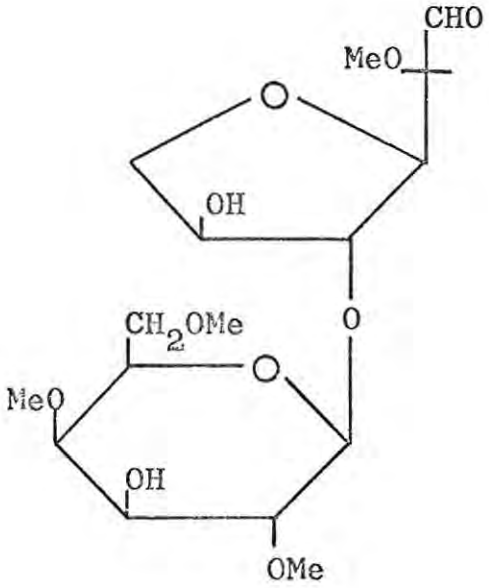
G_L= L-galactose

Disaccharides XI and XIII differ from X and XII respectively only in having a methyl ether group on position 6 of the D-galactose. Furthermore, alkali treatment of X yields agarobiose while XII yields neoagarobiose.

Methylation of alkali treated porphyran [45] gave, on hydrolysis of the polymer, 2,4,6-tri-O-methylgalactose and 3,6-anhydro-2-O-methylgalactose as the only significant products, confirming that the two types of D-linked units and the two types of L-linked units are structurally equivalent, and that there is no possibility of a branched structure. Methanolysis of this same methylated polymer gave the usual derivatives of "tetramethylagarobiose" XIV in 90% yield (corrected for experimental losses). This confirms that the β-configuration of the 1,4-linkage is the same as in agar. Furthermore, the high yield of the tetramethylagarobiose indicates that the polysaccharide chain must consist of strictly alternating 3-linked β-D-galactosyl and 4-linked α-L-galactosyl units.

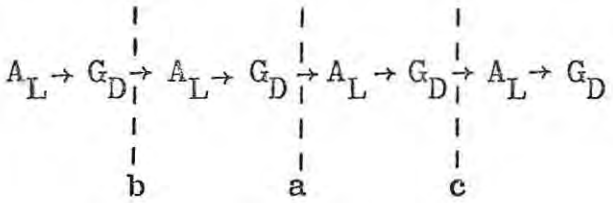
The possibility of portions of the chain having two or more L (or D) units in sequence is further precluded by the absence of oligosaccharides which would have been obtained from such portions of the chain. Rees has termed this a "masked repeating" structure.

XIV



In order to investigate further the linkages in porphyran, Turvey and Christison [46] isolated an enzyme from a Cytophaga species, incubation of which with various galactan sulphates suggested that the enzyme is an agarase i.e. it is specific for 3,6-anhydro-L-galactose residues linked to D-galactose. When this enzyme was applied to porphyran [47] about 70% of the molecule was recovered as high molecular weight material even after prolonged action. Preliminary analysis of the oligosaccharides suggests that the requirements of enzyme specificity largely preclude scission both of the glycosidic linkage between 6-O-methyl-D-galactose and 3,6-anhydro-L-galactose, and of those linkages in which the anhydride is replaced by L-galactose 6-sulphate, the latter giving rise to sulphated oligosaccharides.

In a more recent study Duckworth and Turvey [48] have shown that the highly purified extracellular enzyme from a Cytophaga species has no action on neoagarobiose, neoagarotetraose and their analogues containing 6-O-methyl-D-galactose residues. Furthermore, the preferential scission of neoagaro-octaose XV at position "a" to give two molecules of neoagarotetraose rather than scission at positions "b" or "c" implies that the interior β-D-galactosidic linkages in agarose are hydrolysed faster than those nearest the chain ends.

XV 

A_L = 3,6-anhydro-L-galactose

G_D = D-galactose

Hydrolysis of agarose with this enzyme [49] yielded neoagarotetraose as the preponderant oligosaccharide. This was also the case in the slower enzymic hydrolysis of alkali-treated porphyran, except that almost 50% of the molecule was not degraded. Although in the ideal case the only difference between agarose and alkali-treated porphyran should be the 6-O-methyl groups, in practice the alkali-modified porphyran contained 1.8% sulphate which could not be removed with alkali (but cf. Gracilaria agars [29]). Duckworth and Turvey [49] suggest that it is this small amount of sulphate which prevents enzymic hydrolysis anywhere near these groups.

From the enzymic hydrolysis of native porphyran they isolated the oligosaccharides 6^3-O-methylneoagarotetraose and 6^1-O-methylneoagarotetraose in the ratio 5 : 1. Provided that the distribution of the 6-O-methylgalactose residues in the macromolecule is random, this suggests that the enzyme catalyses the hydrolysis of the 6-O-methyl-D-galactosidic linkage at about one fifth of the rate of the D-galactosidic linkage. The random distribution of the 6-O-methyl groups on the D-galactose residues in the macromolecule is suggested by the even distribution of these two monosaccharide residues between both the neutral and the sulphated oligosaccharides.

The distribution of the sulphate groups in porphyran does not appear to be completely random. Although pure sulphated oligosaccharides were not obtained, nevertheless certain broad features emerge. Thus the average DP of these units was 10 and they contained on average three sulphate groups and two 3,6-anhydrogalactose units. No oligosaccharides were isolated in which only one sulphate group was present: furthermore all the oligosaccharides had at least two 3,6-anhydrogalactose units, suggesting one anhydro unit at the non-reducing end and one at the unit penultimate to the reducing end. All these facts suggest that the sulphate groups occur in blocks in the macromolecule rather than in a random manner.

1.1.3. Laurentia pinnatifida

A galactan similar to porphyran is synthesised by Laurentia pinnatifida [50]. Although evidence was obtained for the inhomogeneity of the polysaccharide, all attempts to

fractionate it were unsuccessful. Acid hydrolysis of the polysaccharide showed the presence of D- and L-galactose (3 : 1 ratio), D-xylose (probably from a separate xylan), 6-O-methyl-D-galactose and D-galacturonic acid, while mercaptolysis yielded the dithioacetals of D- and L-galactose, 6-O-methyl-D-galactose, 2-O-methyl-L-galactose, 3,6-anhydro-L-galactose and a saccharide tentatively identified as 3,6-anhydro-2-O-methyl-L-galactose (accounting for about one third of the total anhydrosugar present).

Partial hydrolysis of the polysaccharide led to the isolation and characterization of L-galactose 6-sulphate and to the tentative characterization of D-galactose 2-sulphate, while other sugar sulphates were also detected. There is also evidence for the existence in the galactan of sulphate ester groups on both 6-O-methyl-D-galactose and 2-O-methyl-L-galactose residues. Further evidence on the location of sulphate ester groups was obtained by alkali treatment of the polysaccharide. Thus the total decrease in the galactose content during desulphation is approximately equal to the L-galactose content before desulphation, suggesting that most of the L-galactose units are 6-sulphated (provided that no D-galactose residues are 6-sulphated, and the authors have good reason to assume that they are not). Furthermore, the decrease in galactose is less than the increase in 3,6-anhydrogalactoses, which is explained thus : the amount of 2-O-methyl-L-galactose decreases during desulphation and the total molar amount of L-galactose and 2-O-methyl-L-galactose (1.20 mol) equals the amount of 3,6-anhydrogalactose (1.21 mol) formed during desulphation (assuming, of course, that the 2-O-methyl-L-galactose units exist

in part as 1,4-linked 2-O-methyl-L-galactose 6-sulphate). The authors consider that the latter sugar is the biological precursor of the 3,6-anhydro-2-O-methyl-L-galactose in the polysaccharide.

Methylation of the sulphated polysaccharide [51] and analysis of its hydrolytic products, together with the evidence cited above for the location of the sulphate groups, suggests that in the galactan sulphate both D-galactose and 6-O-methyl-D-galactose residues are linked through position 3, while the L-galactose and 2-O-methyl-L-galactose residues are linked through position 4. Although the alternating sequence characteristic of the agar-type polysaccharides has not been established in this case, the D : L ratio for all the galactose-derived residues does suggest a basic repeating unit. It would, of course, be largely masked by the ester sulphate and methoxyl groups. Some evidence is given for possible branching in the molecule. The D-galacturonic acid in the polysaccharide gave the 2,4-di-O-methyl ether on methylation indicating that it is 1,3-linked, but no decision could be made as to whether these uronic acids occur as part of the galactan sulphate or whether they occur as a discreet polymer. No information was given on the position of the D-xylose units, but it is possible that they are part of a separate polymer since a xylan was found in the seaweed residue after extraction of the galactan.

1.2. Carrageenan-type polysaccharides

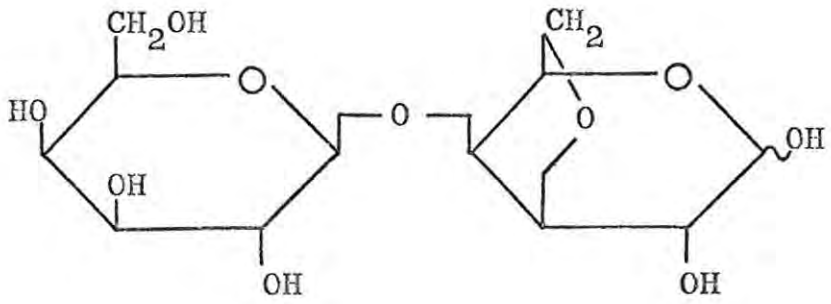
Carrageenan (Irish moss mucilage) is the name given originally to the mucilage of *Chondrus crispus* and *Gigartina stellata*, and although these are still the chief commercial sources, similar polysaccharides are found also in *Iridophycus*, *Hypnea*, *Furcellaria*, *Polyides* and *Eucheuma* species.

Irish Moss mucilage has been subjected to chemical analysis for at least one hundred years : oxidation with nitric acid yielded mucic acid [52], the presence of galactose being confirmed by Tollens *et al* [53]. The surprising discovery by Haas [54] in 1921 that the polysaccharide carries sulphate hemi-ester groups led him to propose the formula $R(OSO_2O)_2Ca$ for the polysaccharide. From methylation and desulphation studies on *Gigartina stellata* Dewar and Percival [55] concluded that the galactose units in carrageenan are galactopyranose residues linked through the 1- and 3-positions and having sulphate hemi-ester groups at the 4-position.

Although the heterogeneity of carrageenan had long been suspected [56,57], it was not until 1953 that the mucilage was fractionated into a number of polysaccharides by the addition of potassium chloride [58,59]. Thus that fraction which gelled at a concentration of 0.15M KCl was termed κ-carrageenan (40%) while the soluble fraction was termed λ-carrageenan (45%).

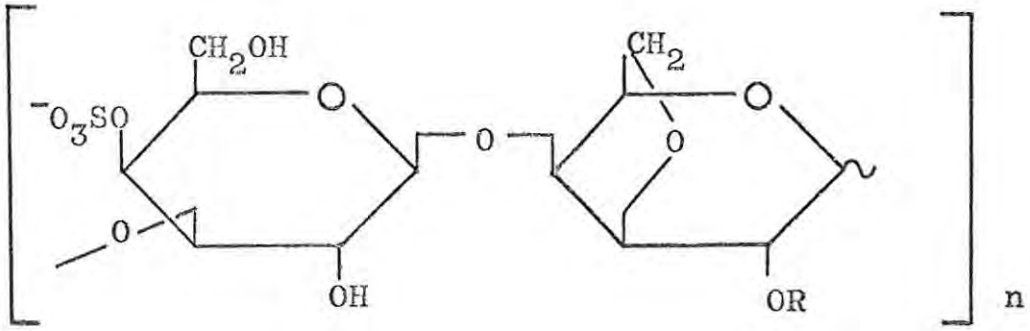
1.2.1. κ- and ι-carrageenan

Partial mercaptolysis of κ-carrageenan by O'Neill [60] gave the diethylmercaptals of D-galactose, 3,6-anhydro-D-galactose, and 4-O-β-galactopyranosyl-3,6-anhydro-D-galactose XVI, the high yield of this carrabiose diethylmercaptal suggesting



XVI : Carrabiose

that it is unlikely that any of the 3,6-anhydro-D-galactose residues occur together in the chain (structure of carrabiose has been proven by synthesis [61]). This evidence indicates that a large part of κ-carrageenan consists mainly of the repeating sequence XVII (A).



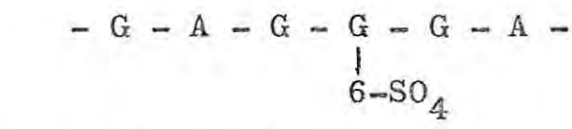
XVII : (A) R = H (B) R = SO_3^- for one seventh and R = H for the remainder.

A macromolecule composed entirely of this repeating carrabiose sequence would have a galactose : 3,6-anhydrogalactose : sulphate ratio of 1 : 1 : 1. However, in his sample of κ-carrageenan O'Neill [62] obtained a ratio of 1 : 0.83 : 1.17 respectively. Furthermore, previous workers had reported some branching in the polysaccharide [63] :

thus in order to explain the above ratio O'Neill suggested that the repeating backbone might be substituted with single unit galactose di-sulphate sidechains linked 1,6 to the galactose residues, with one side chain for every five carrabiose units. Further suggestions as to the structure of κ-carrageenan have been made by other workers [64], but the problem has been largely resolved by Rees et al. These workers showed, by methylation studies [65,66], that all the 3-linked galactose units are 4-sulphated, and that a proportion (probably a quarter to a fifth) of the 3,6-anhydrogalactose units are 2-sulphated. They have also shown [65,67] that a small proportion of the galactose units are not 3-linked, but are 6-sulphated, and that some of these are in addition sulphated at position 2. This is supported by the isolation by Painter [68] of D-galactose 2-, 4- and 6-sulphate and 3,6-anhydro-D-galactose 2-sulphate from the partial acid hydrolysate of κ-carrageenan, while a small amount of a suspected galactose disulphate was also detected. The mode of linkage of these sulphated galactose units and their position in the macromolecule chains was elucidated in the following way [69]. When κ-carrageenan was treated with alkaline borohydride, the formation of 3,6-anhydrogalactose residues in the macromolecule and the simultaneous release of free sulphate [14] changed the galactose : 3,6-anhydrogalactose : sulphate ratio from 1 : 0.89 : 1.22 to 0.95 : 0.95 : 1.17, while the carrabiose content of the polysaccharide increased simultaneously from 88% to 99% (corrected). Methylation of this alkali-modified κ-carrageenan indicated that the structure was not branched. Furthermore, those sulphated galactose residues in the native polymer which may be converted into

3,6-anhydrogalactose are periodate labile and therefore cannot be galactose 3-sulphate. The only way to explain this satisfactorily is to assume that the 6-sulphated galactose residues are 4-linked and that they occur in place of 3,6-anhydrogalactose units at intervals along the alternating chain (XVIII).

XVIII



A= 3,6-anhydrogalactose G= galactose

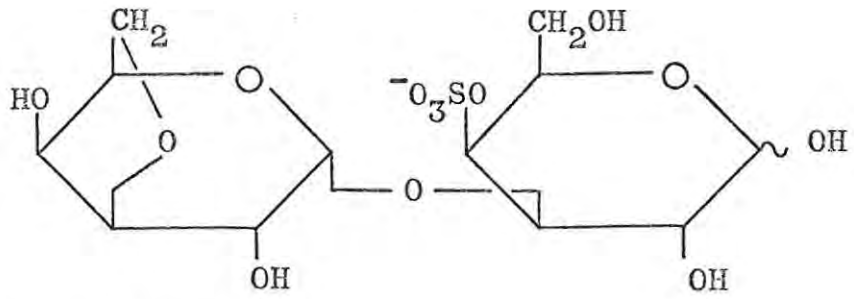
This indicates further that all the 3,6-anhydrogalactose residues are present entirely in carrabiose segments. The low recovery (88%) of carrabiose from the native polymer is explained away, since mild methanolysis cleaves only A→G bonds ; thus for example in the above structure only one anhydro-unit is released as carrabiose, the carrabiose yield being therefore only 50%. Alkaline modification would convert this segment into XIX supporting the evidence that the alkali modified

XIX - G - A - G - A - G - A -

polymer is built up entirely of carrabiose units. The structure of the alkali modified polymer is shown in XVII (B). In the native polymer about 4% of the 3,6-anhydrogalactose is replaced by galactose 6-sulphate and about 1% by galactose 2,6-disulphate, which would correspond to an analytical ratio of 1 : 0.90 : 1.19 (*cf.* above). The structure of κ-carrageenan is thus of the "masked repeating" type (*cf.* porphyran).

Enzymic hydrolysis of κ-carrageenan by Weigl and Yaphe [71] (κ-carrageenase isolated from *Pseudomonas carrageenovora*)

gave a homologous series of sulphated oligosaccharides with neocarrabiose-4-O-sulphate XX as the major degradation product, plus an enzyme resistant fraction. This confirms the position of the sulphate ester and the glycosidic linkage of the galactose and also establishes the configuration of the 1,3-linkage.



XX : neocarrabiose-4-O-sulphate

The enzyme resistant fraction has a higher concentration of sulphate than κ-carrageenan (galactose : 3,6-anhydrogalactose : Sulphate = 1.40 : 1.0 : 1.81 and for native κ-carrageenan = 1.18 : 1.0 : 1.36). Alkali treatment liberates 19% of the sulphate with concomitant formation of a further 14% 3,6-anhydrogalactose. This alkali-treated fraction can be degraded by κ-carrageenase.

Thus the enzyme resistant region appears to be a biogenetically "unfinished" portion of the κ-carrageenan molecule, the 3,6-anhydrogalactose of which may be formed by the desulphation of 6-sulphated galactose units [72].

In order to further investigate the homogeneity of κ-carrageenan, a sample from C. crispus was separated into sub-fractions by graded precipitation from aqueous solution by propan-2-ol [73] (Table II).

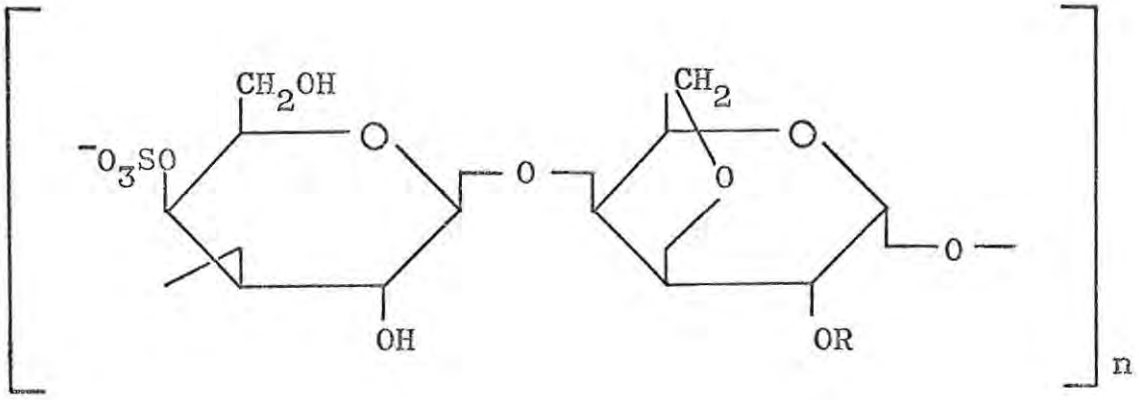
TABLE II : FRACTIONATION OF κ-CARRAGEENAN FROM CHONDRUS CRISPUS

Sample	Source	Analysis $G:A:SO_4^{2-}$	4-linked units (%) which occur as : (i) A	(ii) A-2-SO_4	(iii) G-6-SO_4	(iv) G-2,6-di-SO_4
a	C. Cripus	1 : 0.82 : 1.25	70 - 80	10 - 20	8	0
b	First sub. fr. from a.	1 : 0.89 : 1.07	94	5	1	0
c	Last sub. fr. from a.	1 : 0.67 : 1.20	60	25	13	2

A = 3,6-anhydrogalactose
G = galactose

The first sub-fraction differs markedly from the final one in that the former contains far less 3,6-anhydrogalactose 2-sulphate and galactose 6-sulphate than the latter. The authors thus conclude that κ-carrageenan from this source contains a family of molecules which differ in certain details of structure because certain biological steps such as 3,6-anhydride formation and sulphate formation only occur after polymerisation of galactose to form the alternating 1,3-, 1,4-linked backbone. This supports the above isolation (74) of a high molecular weight enzyme-resistant fraction which is rich in galactose 6-sulphate. Indeed, Rees (75) now defines κ-carrageenan as having the structure XXI (A) where all the 3-linked galactose residues are fully sulphated at position 4, while some of the 4-linked units are galactose 6-sulphate, but most are 3,6-anhydrogalactose, and both are partly 2-sulphated. The structure XXI (B) is defined as ι-carrageenan where all the 3,6-anhydrogalactose residues are 2-sulphated, and 10% of the anhydride is replaced by galactose 2,6-disulphate. ι-Carrageenan can be distinguished from κ-carrageenan by its physical properties (75). Thus it shows a strong band in the infrared spectrum at 805cm^{-1} which is due to 3,6-anhydro-D-

galactose 2-sulphate. Furthermore, ι-carrageenan forms more elastic gels in the presence of K^+ ions than does κ-carrageenan. ι-Carrageenan may be extracted from seaweeds such as Eucheuma spinosum and Agardhiella tenera.



XXI (A) : κ-carrageenan. Mostly R = H : some of the 4-linked units are galactose 6-sulphate, and some are 3,6-anhydrogalactose, and both are partly 2-sulphated.

(B) : ι-carrageenan. R = SO_3^- , but 10% of the anhydride residues are replaced by galactose 2,6-disulphate.

Although the molecular structure of potassium-precipitable carrageenan is so heterogeneous, it bears no relationship to λ-carrageenan since κ-carrageenan and ι-carrageenan do not contain 3-linked galactose 2-sulphate. (Studies on 6 samples of potassium-precipitable carrageenan from various sources show that virtually all 3-linked units are present as galactose 4-sulphate). However there is a relationship between κ-carrageenan and μ-carrageenan (see later).

1.2.2. Structures of λ- and μ-carrageenan

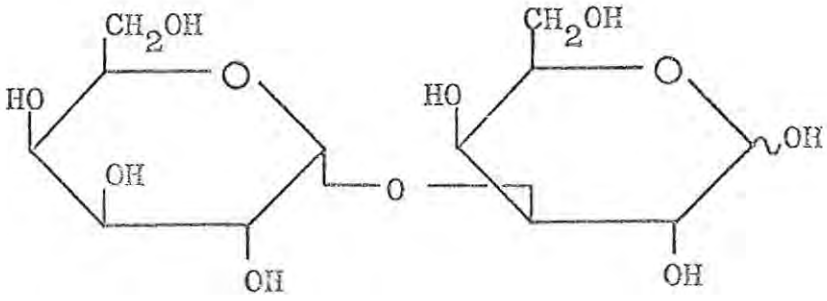
λ-Carrageenan [59] is the name that has long been given to the soluble polysaccharide(s) obtained from a solution of carrageenan after removal of the κ-component with potassium ions.

However, present indications are that this "KCl-soluble" fraction may contain two distinct polysaccharides, depending on the source.

The KCl-soluble component is a highly sulphated galactan (ca. 35% sulphate) comprising D-galactose, 3,6-anhydro-D-galactose and L-galactose. The composition of KCl-soluble carrageenan varies with the source e.g. samples isolated from C. crispus contain little 3,6-anhydro-D-galactose, whereas those isolated from G. stellata contain appreciable amounts of this sugar.

Acetolysis of the KCl-soluble component [76] led to the isolation of 3-O-α-D-galactopyranosyl-D-galactose XXII in high yield. The presence of 1,4-linkages in the polysaccharide is evident from the isolation [77] of carrabiose diethyl dithioacetal XVI (4-O-β-D-galactopyranosyl-3,6-anhydro-D-galactose diethyl dithioacetal) from a partial mercaptolysis of alkali-treated KCl-soluble carrageenan.

XXII

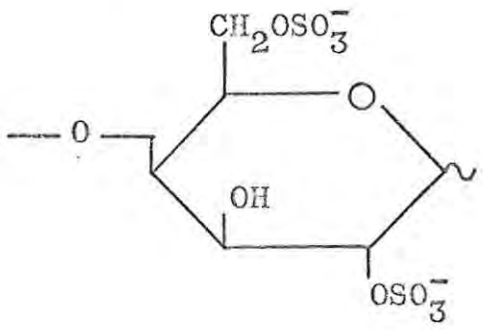


Furthermore, since the alkali-treated KCl-soluble carrageenan closely resembles κ-carrageenan, it is evident that a large proportion of the galactose is 4-linked. The approximate 1 : 1 ratio for 1,3- and 1,4-linkages was established by the

isolation in similar yield of 2,4,6- and 2,3,6-tri-O-methyl-D-galactose from the hydrolysate of methylated desulphated KCl-soluble carrageenan : no evidence for any other linkage (or branching) was obtained.

Since the mode of linkage of the galactose-derived residues is now firmly established, it only remains to establish the position of the sulphate hemi-ester groups. Rees [77] has shown that alkali treatment of KCl-soluble carrageenan (from C. crispus) releases one third of the sulphate groups with concomitant formation of 3,6-anhydro-D-galactose residues. Those units which give rise to the 3,6-anhydrogalactose residues (i.e. the "precursor units") constitute 40% of the structural units and are 1,4-linked (since the only other linkage in the polysaccharide is 1,3). That they can also exist mainly as the 2,6-disulphate XXIII is evident from the following facts [77,78] : (i) 3-O-methyl-D-galactose was isolated as the major component from the hydrolysate of methylated native KCl-soluble carrageenan, which further suggests that,

XXIII

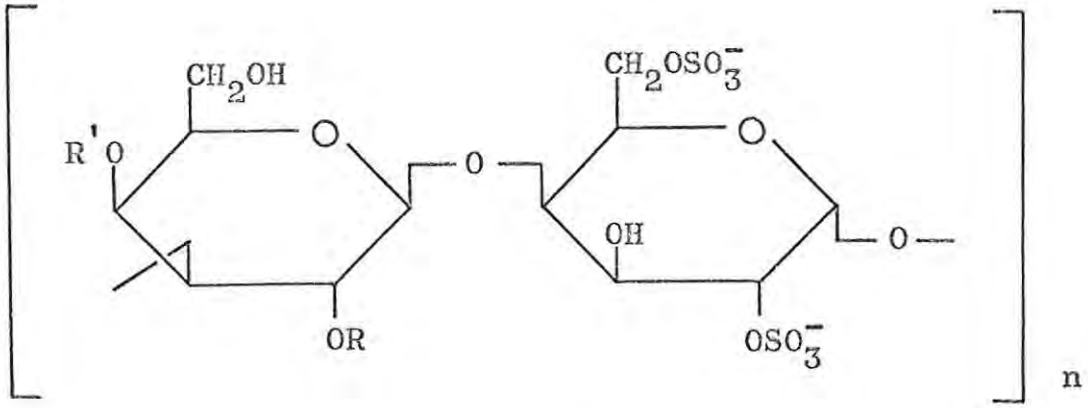


(ii) the sulphate which is released during the formation of the 3,6-anhydrogalactose residues is located mainly at position 6 and not at position 3. (iii) D-galactose 6-sulphate was

isolated from a partial acid hydrolysate of the native polymer. (iv) Since the 1,4-linkages are resistant to periodate oxidation and there is no branching in the polymer, the group protecting the vicinal hydroxyl groups appears to be sulphate at postion 2 (the removal of this acid-labile ester with hot dilute acid leaves the glycol group unprotected, when it can be oxidised with periodate). (v) The evidence for the 2-sulphate was further strengthened by the isolation of a substance with the expected properties of 3,6-anhydrogalactitol-2-sulphate from a Smith degradation of the alkali-treated KCl-soluble polysaccharide. (vi) The rate of removal of the sulphate from the polysaccharide during acidic hydrolysis is consistent with the existence of 2,6-disulphated units.

In order to obtain further evidence for the location of the sulphate ester, the native KCl-soluble polysaccharide was methylated [78]. As already mentioned, the major hydrolysis product was 3-O-methyl-D-galactose which arose from the 1,4-linked 2,6-disulphate units. After correction for the proportion of 6-sulphate, which underwent elimination during the methylation, the yield of the 3-O-methyl-D-galactose corresponded with the estimated proportion of 1,4-linked units in the polymer (i.e. 45 - 58%), indicating a large amount of the 2,6-disulphate. The other methylated saccharides in the hydrolysate were 4,6-di-O-methyl-D-galactose (31%), 2,4,6-tri-O-methyl-D-galactose (13%), and 2,6-di-O-methyl-D-galactose (8%). The 2,6-di-O-methyl-D-galactose is considered to have arisen from 1,3-linked 4-sulphate units rather than 1,4-linked 3-sulphate units, since the former sugar was also present in the hydrolysate of methylated alkali-treated KCl-soluble

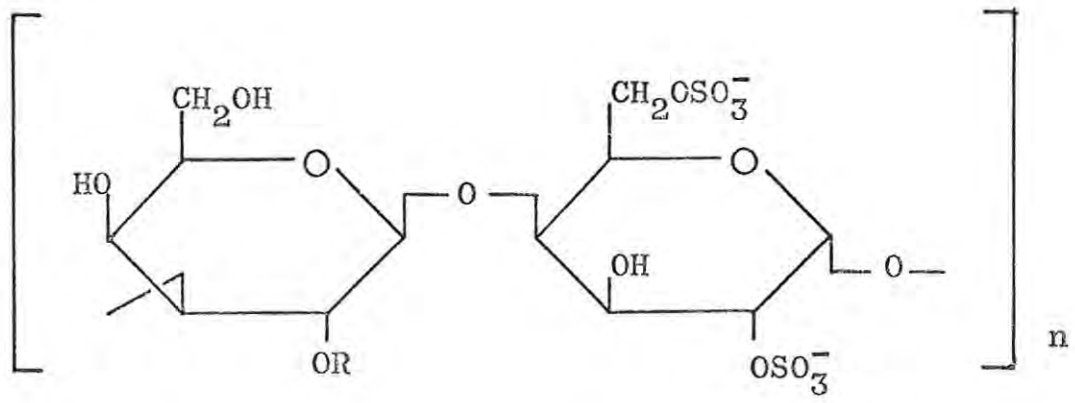
carrageenan. The 2,4,6-tri-O-methyl-D-galactose was obviously derived from 1,3-linked units, while the 4,6-di-O-methyl-D-galactose was derived from 1,3-linked galactose 2-sulphate. The above facts are consistent with structure XXIV for the KCl-soluble component of carrageenan.



XXIV : R usually SO_3^- and less frequently H
When R = SO_3^- , R^1 = H
When R = H , R^1 = usually H and less frequently SO_3^-

A survey of a number of samples of the KCl-soluble component has shown [2,79] that in addition to the above structural components XXIV, some 3,6-anhydro-D-galactose is also present and, when the concentration of this sugar is low, the concentration of 4-sulphate is also low. Dolan and Rees [78] thus consider that the 3,6-anhydro-D-galactose and the D-galactose 4-sulphate are structural features more characteristic of κ- than λ-carrageenan. They therefore define λ-carrageenan as a molecule XXV devoid of these two minor components and consider it as but one extreme of a spectrum of polysaccharides of varying composition (cf. agar). The strictly alternating sequence of 1,3- and 1,4-linkages is indicated by the (corrected) carrabiose content of 95% for alkali-modified λ-carrageenan [65].

XXV : R usually SO_3^- and less frequently H.



The other structural features of the polysaccharide complex (i.e. the 3,6-anhydro-D-galactose and the galactose 4-sulphate) are thus thought to constitute a third component of carrageenan which, although not precipitated by potassium ions, has some structural similarity to κ-carrageenan. In order to investigate this heterogeneity further, the KCl-soluble fraction of C. crispus [80] was treated with alkaline borohydride to convert the 4-linked galactose 6-sulphate residues into 3,6-anhydrogalactose residues XXVI. On addition of KCl to the modified polysaccharide solution, a precipitate P (10%) and a soluble polysaccharide Q (87%) were obtained. Hydrolysis of methylated P gave 2,6-di-O-methylgalactose and 3,6-anhydro-2-O-methylgalactose while hydrolysis of methylated Q gave 4,6-di-O-methylgalactose and 2,4,6-tri-O-methylgalactose but neither of the saccharides that P gave. Methanolysis of both P and Q gave carrabiose dimethyl acetal in such yields as to confirm that the polymers are made up entirely of carrabiose segments.

In Q 70% (from quantitative data) of the galactose units are 2-sulphated, while virtually all the 3,6-anhydrogalactose

KCl-soluble carrageenan (*C. crispus*)

(1) alkali treatment : 4-linked galactose 6-sulphate → 3,6-anhydrogalactose

(2) KCl solution

precipitate : P (10%)

(1) methylate
(2) hydrolyse

2,6-di-O-methyl-galactose
3,6-anhydro-2-O-methyl-galactose

traces { 4,6-di-O-methyl-galactose
traces { 2,4,6-tri-O-methyl-galactose

methanolysis

carrabiose dimethyl acetal in theoretical yield for κ-carrageenan

soluble polysaccharide : Q (87%)

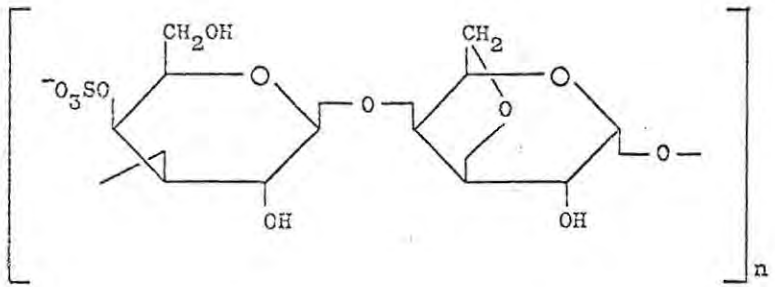
(1) methylate
(2) hydrolyse

4,6-di-O-methyl-galactose
2,4,6-tri-O-methyl-galactose
but none of the major products of P.

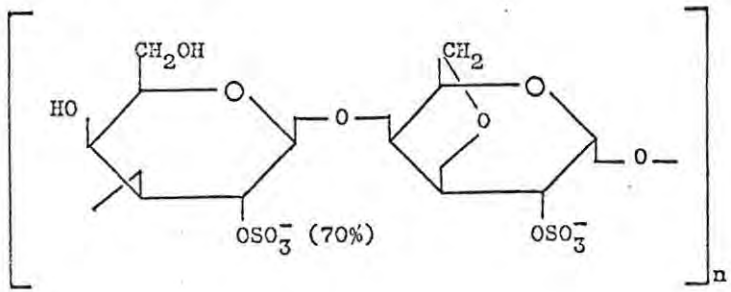
(1) methylate
(2) oxidative hydrolysis

3,6-anhydrogalactonic acid but little of the 2-methyl ether.

Thus P is :



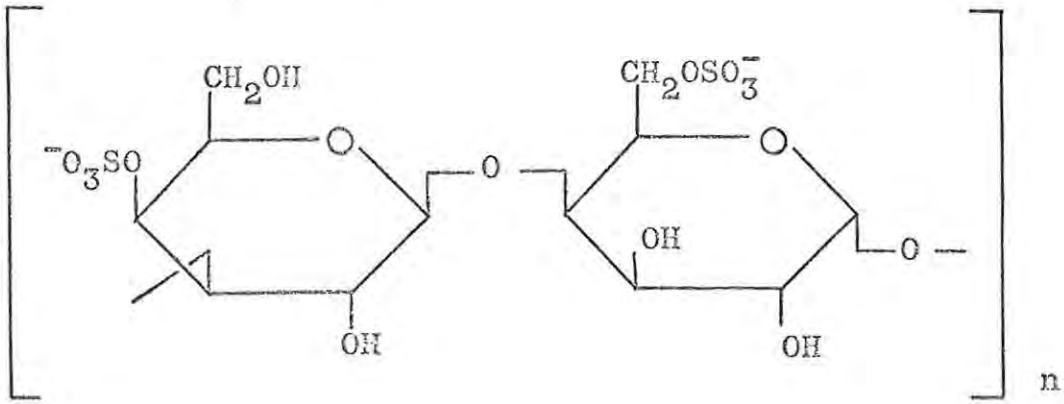
Thus Q is :



XXVI : Structures P and Q are thus of the "masked repeating" type.

residues are sulphated, since oxidative hydrolysis of methylated Q gave 3,6-anhydrogalactonic acid and almost none of the 2-methyl ether. Before modification with alkali, Q therefore corresponds to XXV which is by definition λ-carrageenan.

Since the main hydrolysis product of methylated P is 2,6-di-O-methylgalactose, the galactose residues in P must be largely 4-sulphated, while oxidative hydrolysis gave 3,6-anhydro-2-O-methylgalactonic acid with little 3,6-anhydrogalactonic acid, indicating that the 3,6-anhydride is largely nonsulphated. Furthermore, those samples which gave a high yield of 2,6-di-O-methylgalactose after methylation and hydrolysis and which were therefore rich in the precursor of P, also had high contents of native 3,6-anhydrogalactose. This suggests that the 3,6-anhydride, present before alkali modification of the KCl-soluble fraction, occurs in the precursor of P. The precursor of P thus has the structure XXVII and is termed μ-carrageenan. The replacement of galactose 6-sulphate with 3,6-anhydrogalactose is variable and dependent on the source. Rees [1] has suggested that μ-carrageenan might very well be a biological precursor of κ-carrageenan. μ-Carrageenan has not yet been isolated as such.



XXVII

In order to further prove the above repeating structure for λ-carrageenan, Lawson and Rees [81] subjected KCl-soluble carrageenan from C. crispus (approximating λ-carrageenan in structure since it was low in 3,6-anhydrogalactose) to acetolysis, and isolated the following oligosaccharides : 3-O-α-D-galactopyranosyl-D-galactose, 4-O-β-D-galactopyranosyl-D-galactose, O-α-D-galactopyranosyl-(1→3)-O-β-D-galactopyranosyl-(1→4)-D-galactose, O-β-D-galactopyranosyl-(1→4)-O-α-D-galactopyranosyl-(1→3)-D-galactose, and O-α-D-galactopyranosyl-(1→3)-O-β-D-galactopyranosyl-(1→4)-O-α-D-galactopyranosyl-(1→3)-D-galactose. All these acetolysis products are consistent with a polysaccharide structure having alternately α-1,3- and β-1,4-linked galactose residues.

The relationship between κ- and μ-carrageenan is best illustrated as follows. The presence of galactose 6-sulphate has a marked effect on the gelling strength of κ-carrageenan. Thus the removal of even as little as 0.5% of the sugar with the concomitant formation of 3,6-anhydrogalactose increases the gelling strength markedly in spite of a certain amount of alkaline degradation. This is because removal of the "kinks" caused by the difference in conformation between the galactose 6-sulphate and 3,6-anhydrogalactose increases the gel strength (cf. agar). The gel framework is caused by cross-linking of chains in double helices and 6-sulphate residues represent impediments in the cross-linking i.e. "kinks" in the helix strand. These kinks regulate the extent of helix formation and therefore the physical and biological properties of the macromolecule. Rees has recently [70] isolated an enzyme

from Gigartina stellata, which when applied to μ-carrageenan (essentially κ-carrageenan which is very rich in kinks) resulted in the formation of 3,6-anhydrogalactose to an extent of 14% relative to the native anhydride. It is not clear whether elimination occurs at the 6-sulphate or 2,6-disulphate residues or both (the 2-sulphated units hardly effect the shape of the macromolecule so that the formation of the helix is not hindered). Rees thinks that "if this dekinking occurs in the living plant, it might provide a means of adaption, for example, to increased severity of tidal action".

1.2.3. Other carrageenan-type polysaccharides.

1.2.31. κ-Carrageenan from Gigartina skottsbergii S. et G.

The κ-carrageenan from this seaweed [82,83] was not precipitated diffusely but yielded four distinct sub-fractions at KCl concentrations of 0.1, 0.4, 0.65 and 0.75M. The combined fraction at 0.65 - 0.75M was postulated as containing a structural unit composed of a backbone of four galactose residues, three 3,6-anhydrogalactose residues and five sulphate ester groups (two as 3,6-anhydrogalactose 2-sulphate, two as galactose 4-sulphate and one as galactose 6-sulphate). For the fraction precipitated at 0.3 - 0.4M KCl a structure was postulated which contains, for every ten 3-linked galactose residues, six 4-linked 3,6-anhydrogalactose residues and ten sulphate groups (five as 3,6-anhydrogalactose 2-sulphate and five as galactose 2-sulphate). However, I consider the evidence for these structures to be rather tenuous : e.g. the position of the SO_4 (a key factor in the above structures) is assigned solely on the basis of IR spectra and reaction rates for hydrolysis of 3,6-anhydrogalactose linkages and

sulphate ester linkages, while, for example, no supporting evidence from so powerful a tool as methylation analysis is given (cf. ref [69]). Furthermore, the large proportion of 3,6-anhydrogalactose 2-sulphate in, for example, the 0.65 - 0.75M fraction [82] should give rise to a strong band in the IR at 805cm^{-1} [73] and since only a weak shoulder is present in this region, it is certainly unlikely that 60% of the 3,6-anhydrogalactose residues are sulphated in the 2-position.

1.2.32. Furcellaran.

Furcellaran (Danish Agar) is extracted on a commercial scale from Furcellaria fastigiata. It has $[\alpha]_D$ + 75° and was shown to contain galactose (46%), 3,6-anhydrogalactose, ester sulphate and traces of xylose. Hydrolysis of the methylated desulphated (by acetylation) polysaccharide yielded 2,4-di-O-methyl-, 2,4,6-tri-O-methyl- and 2,3,4,6-tetra-O-methyl-galactose suggesting a backbone of 1,3-linked galactose residues with some branching on C_6 [84]. Painter [85] has further shown the similarity between κ-carrageenan and this polysaccharide by precipitation with potassium chloride. The polymer thus purified had galactose 43%, 3,6-anhydrogalactose 30% and ester sulphate 20% (cf. κ-carrageenan 26%). Partial mercaptolysis [85] gave 4-O-β-D-galactopyranosyl-3,6-anhydro-D-galactose diethyl mercaptal. Autohydrolysis of the polysaccharide [68] afforded D-galactose, and its 2-, 4- and 6-sulphate, 3,6-anhydro-D-galactose and its 2-sulphate, and carrabiose (cf. κ-carrageenan p.24). Large amounts of unidentified sulphated oligosaccharides were also present. Enzymic hydrolysis [86] indicates that 56% of the polysaccharide

is composed of a κ-carrageenan-type polymer. Thus this polysaccharide has a similar but less strictly alternating sequence than κ-carrageenan (XXI (A)), the difference being that only half of the 3-linked galactose residues are 4-sulphated [34].

1.2.33. Gigartina tenella

The polysaccharide extracted from Gigartina tenella was separated into κ-carrageenan and KCl-soluble carrageenan-type polysaccharides by potassium chloride. Hydrolysis and methanolysis of the κ-polysaccharide show the main components to be D-galactose, 3,6-anhydro-D-galactose and ester sulphate in the molar ratio 1 : 0.98 : 1.17 (cf. κ-carrageenan). Small amounts of L-galactose and D-xylose were also obtained, but it has not yet been determined whether or not these were due to contaminants. Partial methanolysis gave a 65% yield of carrabiose dimethyl acetal indicating that the disaccharide constitutes a repeating unit in the polysaccharide.

1.2.34. Hypnea species polysaccharides.

Hypnea specifera yields a sulphated polysaccharide [87] which is precipitated by potassium ions. It afforded D-galactose, 3,6-anhydro-D-galactose and ester sulphate in the molar ratio 1.4 : 1.1 : 1.0. From the results of methylation of both native and desulphated polysaccharides it appears that the galactose is 1,3-linked with hemi-ester sulphate on position 4. The isolation of the disaccharide 3,6-anhydro-4-O-β-D-galactopyranosyl-D-galactose (XVI) from a partial methanolysate of the polysaccharide establishes the

linkage of the 3,6-anhydrogalactose, while the high yield of the disaccharide indicates that the molecule is largely built up of this unit.

Enzymic hydrolysis [86] established the presence of 87% of κ-carrageenan-type material in Hypnea musciformis.

1.2.35. Eucheuma species polysaccharides

The mucilages of Eucheuma muricatum [88] and E. spinosum [89] also contain polysaccharides with properties similar to κ-carrageenan, although that from E. spinosum resembles ι-carrageenan more closely.

1.2.36. Mucilage of Irideae laminarioides

The mucilage of Irideae laminarioides contains a sulphated galactan [90]. Methylation established that the galactan is 1,3-linked [91], while the sulphate was placed at the 6-position [92]. Using the technique of enzymic hydrolysis Yaphe [86] showed that 36% of the polysaccharide consists of κ-carrageenan-type material.

1.3. Grateloupiaceae Polysaccharides

1.3.1. Aeodes orbitosa

Hot water extraction of this seaweed yielded a highly sulphated (27.5% sulphate) polysaccharide, aeodan [93], which was shown to contain D- and L-galactose, 2-O-methyl-D-galactose, 4-O-methyl-L-galactose [94], 6-O-methyl-D-galactose, D-xylose and glycerol, the ratio galactose : mono-O-methylgalactose : sulphate ester being 12 : 2 : 9. Alkali treatment liberated 16% of the sulphate with concomitant formation of 5% 3,6-anhydrogalactose,

indicating that most of the 1,4-linked residues are sulphate-free. Native aeodan consumed 0.21 moles periodate per sulphate-free anhydrohexose unit indicating (together with further evidence stated hereunder) a large number of 1,3-links in the macromolecule. Hydrolysis of the resulting oxopolysaccharide showed that galactose and the O-methylgalactoses had survived the oxidation ; there was no trace of xylose indicating that it is 1,2- or 1,4-linked. The 6-O-methylgalactose is thus 3-linked.

Hydrolysis of methylated desulphated aeodan afforded 2,3,4,6-tetra-O-methylgalactose, 2,3,6- and 2,4,6-tri-O-methyl-D-galactose (major fraction) and some di-O-methylgalactoses. The high yield (80%) of the two tri-O-methylgalactoses indicates the predominance of 1,3- and 1,4-linkages in aeodan. From the results of the hydrolysis of methylated native aeodan the authors [93] suggest that some of the 1,3-linked units are sulphated at position 2 (since 4,6-di-O-methyl-D-galactose is present in this hydrolysate but little is present in the hydrolysate of methylated desulphated aeodan).

From a partial hydrolysis of aeodan were isolated 4-O-β-D-galactopyranosyl-D-galactose, 3-O-D-galactopyranosyl-D-galactose [94] and 4-O-β-D-galactopyranosyl-L-galactose [95], thus establishing the configuration of some of the linkages in aeodan.

1.3.2. Phyllymenia cornea

The highly sulphated (20%) polysaccharide, phyllymenan, from Phyllymenia cornea [96], contains D-galactose, 2-O-methyl-D-galactose, 6-O-methyl-D-galactose, ester sulphate and smaller

amounts of L-galactose, 4-O-methyl-L-galactose, 3,6-anhydrogalactose, xylose and pyruvic acid (isolated as the 2,4-dinitrophenylhydrazone), the proportions being galactose, 43% and total mono-O-methylgalactoses 26%. Very little of the sulphate is alkali-labile. Periodate oxidised only 0.16 mole of sulphate-free anhydrohexose units. This suggests (with evidence cited later) a large proportion of 1,3-links in the macromolecule. Reduction of periodate by native phyllymenan, desulphated phyllymenan and partially desulphated phyllymenan was approximately the same in each case, indicating that desulphation of the polymer does not produce new α-glycol groups. Since the sulphate is not alkali-labile, this indicates that only those residues which are 1,3-linked carry sulphate. The presence of 6-O-methylgalactose in the oxopolysaccharide indicates that it is 1,3-linked.

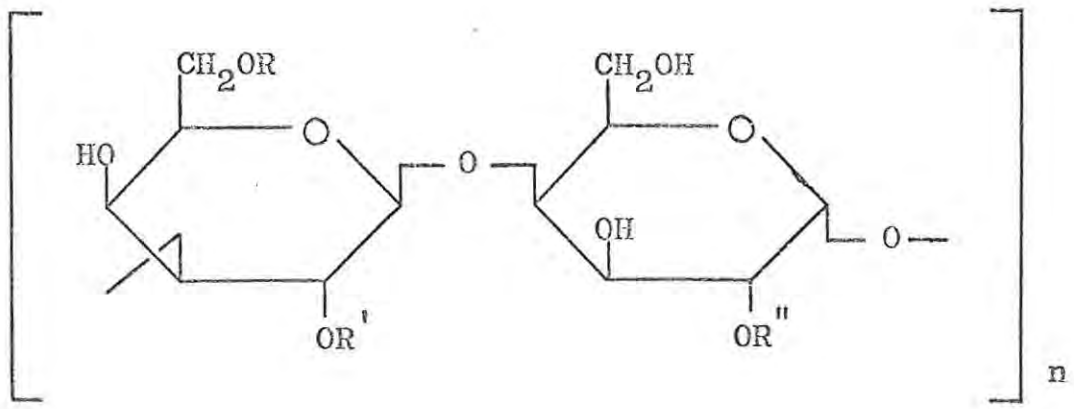
Partial hydrolysis of phyllymenan afforded 4-O-β-D-galactopyranosyl-D-galactose, 4-O-β-D-galactopyranosyl-2-O-methyl-D-galactose (in high yield), 3-O-α-(2-O-methyl-D-galactopyranosyl)-D-galactose and 4-O-(6-O-methyl-D-galactopyranosyl)-2-O-methyl-D-galactose, thus establishing the configuration of some of the glycosidic linkages in the polymer.

From the hydrolysate of methylated desulphated phyllymenan [97] were obtained 2,3,4,6-tetra-O-methylgalactose, 2,4,6- and 2,3,6-tri-O-methyl-D-galactoses in the molar ratio of 1.6 : 10.1 : 8.7. When combined with the above facts, these results can be interpreted as follows. The 2,3,6-tri-O-methyl-D-galactose is considered to have arisen from 1,4-linked D-galactose and 1,4-linked 2-O-methyl-D-galactose residues in

the polymer. The 2,4,6-tri-O-methyl-D-galactose is considered to have arisen from 1,3-linked D-galactose and 1,3-linked 6-O-methyl-D-galactose residues in the polymer.

Hydrolysis of methylated native phyllymenan afforded 2,3,4,6-tetra-, 2,3,6- and 2,4,6-tri-, 2,6- and 4,6-di-, and 2- and 4-mono-O-methyl-D-galactose. Treating these results with some caution the authors [97] conclude the following . The 4,6-di-O-methylgalactose could only have arisen from 1,3-linked D-galactose 2-sulphate. The 2,4,6-tri-O-methylgalactose indicates that not all the 1,3-links are sulphated. The 2,3,6-tri-O-methylgalactose comes from 1,4-linked D-galactose and 2-O-methyl-D-galactose residues in phyllymenan. The mono-O-methylgalactoses and D-galactose are probably due to undermethylation, although some of the 2-O-methyl-D-galactose could have arisen from pyruvic acid attached as the 4,6-acetal to 1,3-linked D-galactose.

On the basis of the above evidence the partial structure XXVIII is presented.



XXVIII : Where :

R = H and less frequently CH_3.

R' = SO_3^- and less frequently H.

R'' = CH_3 and less frequently H.

The linear alternating sequence is shown, not because an alternating sequence has been conclusively proved, but because of the presence of approximately equal proportions of 1,3- and 1,4-linkages in desulphated phyllymenan, and because the isolation of the trisaccharide O-β-D-galactopyranosyl-(1→4)-O-α-(2-O-methyl-D-galactopyranosyl)-(1→3)-D-galactose indicates that at least some regions of alternating structure do exist.

1.3.3. Pachymenia carnosa

Pachymenia carnosa mucilage was shown to contain [98] D-galactose, 2-O-methyl-D-galactose, 6-O-methyl-D-galactose, 4-O-methyl-D- and L-galactose, xylose and ester sulphate (30%) in the molar ratio 6.1 : 1.0 : 0.5 : 0.2 : 0.03 : 6.6. Only 1% 3,6-anhydrogalactose was formed on alkali-treatment of the polysaccharide.

Partial hydrolysis of the polysaccharide gave the following disaccharides : 4-O-β-D-galactopyranosyl-D-galactose, 4-O-β-D-galactopyranosyl-2-O-methyl-D-galactose, 3-O-α-(2-O-methyl-D-galactopyranosyl)-D-galactose, 4-O-β-(6-O-methyl-D-galactopyranosyl)-D-galactose, 4-O-β-(6-O-methyl-D-galactopyranosyl)-2-O-methyl-D-galactose, and (2-O-methyl-galactosyl)-6-O-methylgalactose.

The position of some of the sulphate residue is proved by the isolation of D-galactose 6-sulphate from the acidic fragments of the partial hydrolysate.

The main products from the hydrolysis of the methylated desulphated polysaccharide were 2,3,6- and 2,4,6-tri-O-methyl-D-galactose in approximately 1 : 1 ratio, supporting the above evidence for 1,3- and 1,4-linkages in the polysaccharide.

The relatively large amount of 2,3,4,6-tetra-O-methylgalactose is considered to have arisen from the short degraded chains in the desulphated molecule, while the small amount of di-O-methylgalactoses present are considered to be undermethylation products.

Periodate oxidation studies (combined with the partial hydrolysis evidence above) indicate that the 6-O-methyl-D-galactose is 1,3-linked, and that the 2-O-methyl-D-galactose is 1,4-linked. All the 4-O-methylgalactose is cleaved by periodate (cf. Aeodes ulvoidea [100]). Those galactose units which are 1,3-linked are obviously also immune to periodate.

Acetolysis of the polysaccharide [99] led to the identification of 16 oligosaccharides, including those already mentioned in the partial hydrolysate. All seven trisaccharides were found to contain both α-1,3- and β-1,4-linkages, and the only tetrasaccharide had alternating α-1,3- and β-1,4-linkages. Since methylation studies on the desulphated polysaccharide indicate equal quantities of 1,3- and 1,4-links, the evidence for an alternating sequence is quite strong.

1.3.4. Aeodes ulvoidea

The sulphated polysaccharide from Aeodes ulvoidea [100] was shown to contain D-galactose, 4-O-methyl-L-galactose and 2-O-methyl-D-galactose in the molar ratio 10.5 : 1.1 : 1.0, together with traces of 6-O-methyl-D-galactose, xylose and mannose. This polysaccharide thus differs from the other Gratelouniaceae polysaccharides in that the 4-O-methyl-L-galactose is a major constituent. The susceptibility of this saccharide to oxidation by periodate indicates that it is either present as non-reducing end-group or else 1,6-linked :

the latter possibility being ruled out however by the absence of 2,3,4-tri-O-methylgalactose in the hydrolysate of methylated desulphated polysaccharide. On the other hand, the presence of 2,3,4,6-tetra-O-methyl-L-galactose (1 : 1 ratio with the D-isomer) in the same hydrolysate supports the case for end-group linkage of the 4-O-methyl-L-galactose (no other L- sugar has been found in the polysaccharide).

Comparison of the ratios of D-galactose : 2-O-methyl-D-galactose in the polysaccharide before and after oxidation by periodate, together with the fact that no 2,3,4-tri-O-methylgalactose was found in the hydrolsate of methylated desulphated polysaccharide, indicates that the majority of the 2-O-methyl-D-galactose residues must be either 1,3- and/or 1,4-linked. The low reduction of 0.3 mole of periodate per anhydrohexose unit by all the 4-O-methyl-L-galactose residues and some of the D-galactose residues suggests that at least one half of the D-galactose residues are 1,3-linked and/or a large proportion of the galactose residues contain other glycosidic linkages so as to render them immune to periodate. The low percentage of alkali-labile sulphate in the polysaccharide precludes the possibility of a large proportion of 1,4-linked D-galactose residues protected from periodate attack by sulphate hemi-ester groups at positions 2 and/or 3.

Hydrolysis of the methylated desulphated polysaccharide yielded 2,3,6- and 2,4,6-tri-O-methyl-D-galactose as the major components, indicating a large proportion of 1,4- and 1,3-linkages respectively in the polysaccharide. The small amount of 2,3,4,6-tetra-O-methyl-D-galactose obtained is considered to have arisen mainly from D-galactose and possibly

some 2-O-methyl-D-galactose end groups. In addition, trace quantities of 2-O-methylgalactose and some di-O-methylgalactoses were obtained.

1.4. Miscellaneous Polysaccharides

1.4.1. Anatheca dentata

Hot water extraction of Anatheca dentata afforded a highly sulphated (35%) polysaccharide [101], which was shown to contain D- and L-galactose (ratio 1.6 : 1), D-xylose and ester sulphate in the molar ratio 6.0 : 1.0 : 6.8 respectively. Small amounts of pyruvic acid and D-glucuronic acid were also obtained. The mode of linkage of the galactose was elucidated by the isolation of the following methylated saccharides from the hydrolysis of methylated desulphated polysaccharide : 2,3,4,6-tetra-O-methyl-D- and L-galactose, 2,3,6-tri-O-methyl-L-galactose (with a little of the D-isomer) and 2,4,6-tri-O-methyl-D-galactose (the two tri-O-methylgalactoses being in 2 : 1 ratio). This indicates that most of the L-galactose and some of the D-galactose residues are 4-linked, while unbranched D-galactose residues are 3-linked : also both D- and L-galactose may occur as non-reducing end-group. The xylose gave only 2,3,4-tri-O-methylxylose indicating that all the xylose is present as non-reducing end-group. This applies also to the uronic acid since only 2,3,4-tri-O-methyl-D-glucuronic acid was obtained : furthermore 4-O-α-D-glucuronosyl-L-galactose was isolated from the acidic fragments of a partial hydrolysate of the native polysaccharide.

From these acidic fragments the following pyruvate containing saccharides were isolated : 4,6-O(1'-carboxyethlidene)-

D-galactose and 4-O-β-[4,6-O(1'-carboxyethylidene)]-D-galactopyranosyl-L-galactose, indicating the same site for the pyruvic acid as in agar VII. The presence of pyruvate in this polysaccharide as well as in agar and members of the Grateloupiaceae (Aeodes orbitosa [101] and Aeodes ulvoidea [102]) suggests that pyruvate may well be more widespread in the red seaweed polysaccharides than has previously been thought.

The sulphate hemi-ester groups in the polysaccharide are essentially non-alkali-labile and some were placed by the isolation of L-galactose 3- and 6-sulphate. Since all the L-galactose residues are 1,4-linked this 3- or 6-sulphate would be alkali-labile, and since the polysaccharide does not contain alkali-labile sulphate, those L-galactose residues which carry the sulphate must be either the site of branch-points in the macromolecule or else trisulphated. In support of this, periodate oxidation of the native polysaccharide cleaves 10% of the galactose residues which are therefore 1,4-linked and not sulphated at position 6, while in the desulphated polysaccharide periodate cleaves about half of the galactose residues, showing that during desulphation of the polysaccharide sulphate must have been removed from the 1,4-linked residues at either position 2 or 3 or else from both positions. It is also interesting to note that the molar ratio of L-galactose to sulphate ester groups in the polysaccharide is 1 : 3. Thus the evidence for trisulphated L-galactose in the macromolecule is very strong.

Further evidence for the mode of linkage of the galactose in the macromolecule was obtained by the isolation of the following oligosaccharides from a partial hydrolysate : 4-O-β-D-galactopyranosyl-L-galactose, 3-O-α-L-galactopyranosyl-

D-galactose, 4-O-β-D-galactopyranosyl-D-galactose, O-β-D-galactopyranosyl(1→4)-O-α-L-galactopyranosyl(1→3)-D-galactose, and O-β-D-galactopyranosyl(1→4)-O-α-L-galactopyranosyl(1→3)-O-D-galactopyranosyl(1→4)-L-galactose. Thus a substantial part of the molecule is made up of α-1,3-D- and β-1,4-L-galactose residues. The presence of 4-O-β-D-galactopyranosyl-D-galactose is not surprising since there are more D- than L-galactose residues in the polysaccharide, and thus D-galactose must replace some L-galactose in the saccharide sequence.

Although a repeating sequence for the macromolecule as a whole has not been proven, nevertheless the above results indicate that at least some parts of the macromolecule resemble agar in having a repeating sequence of α-1,3-D- and β-1,4-L-galactose residues, although the polysaccharide resembles λ-carrageenan in respect of its high sulphate content and absence of 3,6-anhydro-galactose. It is interesting that Anatheca dentata belongs to the same family (Solieriaceae) as the Eucheuma species discussed above and which structurally resemble the carrageenans.

1.4.2. Corallina officinalis.

Decalcification of the calcareous red alga Corallina officinalis [103] led to the isolation of a sulphated polysaccharide consisting of galactose, D-xylose and ester sulphate in the molar ratio of 4.2 : 1.9 : 1.0. Mild hydrolysis yielded two galactose monosulphates viz. L-galactose 6-sulphate and galactose 4-sulphate. The polysaccharide contains no 3,6-anhydrogalactose. Periodate oxidation of the polysaccharide indicates that only a few galactose residues are

3-linked, since most of the L-galactose residues and about two-thirds of the D-galactose residues are oxidised by periodate. The xylose residues are all oxidised by periodate and must therefore be linked through either position 2 or 4.

1.4.3. Dilsea edulis

The sulphated galactan from Dilsea edulis [104] ($[\alpha]_D$ + 47°) contained 20 - 24% sulphate. Methylation of the desulphated polysaccharide [105] gave a high yield of 2,4,6-tri-O-methyl-, some 2,3,4,6-tetra-O-methyl-, and a trace of 2,3,6-tri-O-methyl-D-galactose indicating a 1,3-linked galactose backbone. This was confirmed by the oxidation of only 0.2 moles of galactose residues by periodate. Hydrolysis of the mucilage [106] yielded D-galactose, xylose, glucurone, ester sulphate and traces of 3,6-anhydro-D-galactose and hydroxymethylfurfuraldehyde, the latter as a degradation product of the anhydride. From repeated Barry degradation [107] on the polysaccharide the authors [106] concluded that the xylose is 1,3-linked, and that it and the glucurone are probably present in peripheral side-chains : also, some of the galactose is 1,4-linked and also occurs in side-chains. Rees [108] has more recently shown that there exist two dissimilar regions of the polysaccharide which may be in the same or in different molecules. The major component is a chain of 1,3-linked galactopyranose residues, some of which carry sulphate at position 4. The other region consists of an alternating sequence of 1,3- and 1,4-linked galactopyranose residues, with some of the 1,3-linked units perhaps carrying sulphate at position 4 and some of the 1,4-linked units occurring as the 6-sulphate or 3,6-anhydride. He considers the xylose to be

part of a contaminating polysaccharide.

1.4.4. Dumontia incrassata

The polysaccharide [109] has an $[\alpha]_D$ + 53° (cf. + 47° for D. edulis) and forms a highly viscous solution but does not gel. The mucilage contains galactose, sulphate and a uronolactone in the ratio of 9 : 4 : 1. Periodate oxidises one unit in 6 i.e. 5 units are 1,3-linked for every unit not so linked. The ratio of sulphate to sugar (2 : 5) in this polysaccharide is twice as high as in D. edulis (1 : 5).

1.4.5. Funoran

The mucilage of Gloiopeltis furcata has $[\alpha]_D$ - 21°, contains 19% sulphate and forms a viscous solution but does not gel. Methanolysis [110] yielded 3,6-anhydro-L-galactose dimethyl acetal and methyl D- and L-galactosides in the molar ratio of 8 : 12 : 1. Partial methanolysis [111] afforded, in addition, agarobiose dimethyl acetal (in very high yield) and a trace of methyl D-xyloside. Thus the authors are of the opinion that if one disregards the sulphate ester groups, then agarobiose is the chief repeating unit of the mucilage molecule. This polysaccharide may therefore approach the third extreme of the Agar polysaccharide [31] complex. The xylose is considered to be due possibly to a contaminating xylan.

1.4.6. Polysiphonia fastigiata

The hydrolysate of the mucilage of Polysiphonia fastigiata [112] afforded D- and L-galactose (ratio 2 : 1), 6-O-methyl-D- and L-galactose (ratio 9 : 7) and 3,6-anhydro-D- and L-galactose (ratio 1 : 1). A sugar sulphate was also

obtained which was tentatively identified as a mixture of D- and L-galactose 6-sulphate. From the methylated polysaccharide a mixture of 2,4-di-O-methyl-D- and L-galactose was obtained as the chief hydrolytic product. The di-O-methylgalactose probably arose from 3-linked galactose 6-sulphate residues. No further work has been published on this complex polysaccharide.

2. EXPERIMENTAL

Evaporation of solutions was carried out below 45° at reduced pressure using a rotary film evaporator. Specific rotations were measured in water, most of them on a Perkin-Elmer 141 automatic polarimeter. Paper chromatography was carried out on Whatman No. 1 paper, using the following solvent systems: (1) ethyl acetate : acetic acid: formic acid: water (18 : 3 : 1 : 4), (2) butan-1-ol : pyridine : water (9 : 2 : 2), (3) butan-1-ol : ethanol : water (40 : 11 : 9), (4) butanone saturated with water containing 1% 0.88 ammonia, (5) ethyl acetate : pyridine : water (8 : 2 : 1) and (6) butanone : ethanol : water (9 : 1 : 2) saturated with boric acid [81]. R_{gal} values refer to chromatographic mobilities relative to galactose in solvent 1, while R_{gg} values refer to chromatographic mobilities relative to 4-O-β-D-galactopyranosyl-D-galactose in solvent 6. Thin-layer chromatography (TLC) was carried out on glass plates coated with silica gel G containing calcium sulphate as binder, employing butanone : water (85 : 7) as solvent. R_{TMG} values of methylated sugars refer to the rates of travel of methylated sugars relative to 2,3,4,6-tetra-O-methyl-D-galactose on thin layer plates. The spray reagents used for paper and thin-layer chromatography were (a) p-anisidine hydrochloride [113], (b) aniline : diphenylamine : phosphoric acid [114], (c) 10% sulphuric acid in ethanol and (d) triphenyltetrazolium chloride [115]. Gas-liquid chromatography (GLC) for the quantitative determination of sugars as their alditol acetates, was carried out on a Beckman GC-4 gas chromatograph equipped with dual flame-ionization detectors and nitrogen as carrier gas. The column packing used was

20% Apiezon M supported on Chromosorb W (80 - 100 mesh, acid washed and dimethylchlorosilane treated) at an operating temperature of 175°. GLC of the methyl glycosides of the methylated sugars was carried out on a Perkin-Elmer 900 gas-chromatograph using a flame ionization detector and nitrogen as carrier gas. The column packing used was 20% butan-1,4-diol succinate polyester (Applied Science HIEFF - 4BP) on Gaschrom P (80 - 100 mesh) at an operating temperature of 190°. Retention times T are relative to that of methyl 2,3,4,6-tetra-O-methyl-β-D-glucopyranoside. The T values obtained for standard saccharides were methyl 2,3,4,6-tetra-O-methylgalactosides (T 1.61), methyl 2,3,6-tri-O-methylgalactosides (T 2.80, 3.42, 3.66 and 3.95), methyl 2,4,6-tri-O-methylgalactosides (T 3.55 and 3.97), methyl 2,3,4-tri-O-methylgalactosides (T 5.87) and methyl 2,3-di-O-methylgalactosides (T 8.38 and 11.44). Values given in parentheses are for peaks which are incompletely resolved and therefore assigned to more than one component. Infrared spectra were recorded on a Beckman IR - 8 spectrophotometer using KBr discs. Sulphate determinations were carried out using the 4'-chlorobiphenyl-4-ylamine method [116, 117].

Aeodes ulvoidea is easily recognised by its broad, flat fronds which often have an endophyte (belonging to the green algae) growing on them, and, during the processing of the weed, great care was taken to remove all visible traces of this endophyte so as to ensure as homogeneous a product as possible.

2.1. Isolation and purification of the polysaccharide

Wet Aeodes ulvoidea (2.5 kg) was half covered with water, and glacial acetic acid was added to pH 3-4. Steam was passed into the mixture, the acetic acid aiding in the complete disintegration of the weed. The pH rose to between 6 and 7 during the process. The extract was centrifuged hot, and the product was precipitated with ethanol (5 volumes). The crude polysaccharide was washed with ether and dried at 50° under reduced pressure (10% yield on a wet-weight basis). Purification of the polysaccharide was effected by dissolution in water, centrifugation, and precipitation with ethanol. After three such cycles, the polysaccharide was washed with ether and dried at 50° under reduced pressure (Found : $[\alpha]_D^{23}$ + 34°; 3,6-anhydrogalactose [118], 1.4; N,0.4; OMe,2.07; SO_4^{2-} (ref.119), 19.9%). Both the sodium and the ammonium salts of the polysaccharide gave a peak in the infrared at 1240cm^{-1}; only a broad shoulder was present in the 800 - 860cm^{-1} region [120].

2.2. Separation and characterization of the components of the polysaccharide

The polysaccharide (20 g) was hydrolysed with 0.5M sulphuric acid (100 ml) on a boiling water-bath overnight. The solution was neutralised ($BaCO_3$), centrifuged, and evaporated to a hygroscopic, solid foam (11.6 g). Paper chromatography (solvents 1, 3, and 4) of the solid revealed the presence of three sugars with R_{gal} 2.15, 1.75 and 1.00 (solvent 1), corresponding to 2-O-methylgalactose, 4-O-methylgalactose and galactose (major sugar), respectively. The solid was applied to a cellulose column (54 x 430 mm), and the monosaccharides were eluted with butan-1-ol : water (95 : 5). Fractions (50 ml) were analysed by paper chromatography and combined into six major fractions.

Fraction I. The syrup (274 mg), after recrystallisation from ethyl acetate : methanol, yielded 2-O-methyl-D-galactose, m.p. and mixed m.p. 147-148°; $[\alpha]_D^{18}$ + 52 (5 min) → + 92° (*c* 0.58); lit.[93] m.p. 148-149°, $[\alpha]_D^{16}$ + 84.9° (final) (*c* 0.53). The infrared spectrum of this sugar was identical with that of authentic 2-O-methyl-D-galactose. Demethylation [121] of the sugar (5 mg) by heating on a boiling water-bath with 46% HBr (1 ml) gave (paper chromatography) galactose and a trace of 2-O-methylgalactose. Later investigation of the mother liquor from which the 2-O-methyl-D-galactose had crystallised showed the presence of a trace of 6-O-methylgalactose (paper chromatography, solvents 1 and 5, and sprays *a* and *d*).

Fraction II. A syrup (440 mg) which was shown by paper chromatography to be a mixture of 2-O-methylgalactose and 4-O-methylgalactose (solvents 1, 2 and 4).

Fraction III. The syrup (370 mg) was chromatographically identical with 4-O-methylgalactose (solvents 1, 2 and 4). After recrystallisation from methanol, it yielded large, colourless crystals of m.p. 200-202°; $[\alpha]_D$ - 65 (5 min)→ - 83° (c 0.42). The m.p. was depressed to 192° on admixture with 4-O-methyl-D-galactose. Araki et al [26] reported m.p. 202-203°; $[\alpha]_D^{13}$ - 74.8 (27 min)→ - 85.1° (24 h) (c 2.70), for authentic 4-O-methyl-L-galactose. Demethylation [121] gave (paper chromatography) galactose and a trace of starting material. The infrared spectrum of the sugar was identical with that of its enantiomorph. The derived 4-O-methyl-N-phenyl-L-galactosylamine had m.p. 167.5-168°; lit. [26] m.p. 167-168°.

Fraction IV. A syrup (92 mg), shown by paper chromatography (solvents 1, 2 and 4) to contain traces of galactose, mannose (R_{gal} 1.30), xylose (R_{gal} 1.65) and 4-O-methylgalactose (R_{gal} 1.67).

Fraction V. An amorphous solid (4.7 g) which, after recrystallisation from ethanol, yielded D-galactose, m.p. and mixed m.p. 160-162°, $[\alpha]_D^{17}$ + 116 (5 min)→ + 85° (c 0.52). The sugar was oxidized by heating for 1 h with nitric acid (1 ml of 1 : 1 nitric acid : water) at 100° to give mucic acid, m.p. and mixed m.p. 210-212°.

Fraction VI. This fraction, a hygroscopic solid (2.7 g), was eluted from the column with aqueous ethanol and ethanol, and not further investigated.

2.3. Quantitative determination of the hexose residues in the polysaccharide

The method used for the quantitative determination of the sugar residues in the polysaccharide, desulphated polysaccharide and periodate oxidized polysaccharide was similar to that used by Bowker and Turvey [122]. To polysaccharide (50 - 100 mg) was added a known quantity (1 - 2 ml) of a standard erythritol solution (7.34 mg/ml) and 0.5M sulphuric acid (10 ml). The solution was heated on a boiling water-bath for 6 h, cooled, neutralised with saturated barium hydroxide solution to pH 5-6 and centrifuged. Sodium borohydride (200 mg) was added and the solution allowed to stand for 24 h. It was then neutralised carefully with 0.5M sulphuric acid, evaporated to a solid and dried over phosphorus pentoxide at $30^{\circ}/0.1$ mm. Acetylation mixture (5 ml) containing acetic anhydride (50 ml) and sulphuric acid (1 ml) was added to the solid and heated at 80° for 6 h. The solution was cooled, neutralised with sodium bicarbonate to pH 5 and extracted twice with chloroform (10 ml). The chloroform layer was dried ($MgSO_4$), and after suitable adjustment of the concentration, 1 µl samples were injected into the gas chromatograph. Since the response of the detector varies from one glycitol acetate to another, the molar response of the glycitol acetate of each sugar

present in the polysaccharide was determined relative to erythritol acetate. This was done by reducing a solution of a known weight of erythritol and a known weight of the sugar to be determined with sodium borohydride, and then acetylating as outlined above. The concentration of each sugar was estimated from its peak area, i.e. peak height x width at half height.

2.4. Desulphation of the polysaccharide

Polysaccharide (16 g) and 0.15M methanolic hydrogen chloride [123] (500 ml) were shaken for 48 h. Insoluble material was then filtered off, washed with a little dry methanol, and re-treated with fresh, 0.15M methanolic hydrogen chloride (500 ml). The insoluble material (11.0 g) was filtered off, washed with ethanol and dried at 55°/ 0.1 mm (Found: N, 1; SO_4^{2-}, 6.2%). The combined, methanolic filtrates were neutralised (Ag_2CO_3), centrifuged, and evaporated to a syrup. Paper chromatography of a neutralised, acid hydrolysate showed the presence of galactose, 4-O-methylgalactose, and 2-O-methylgalactose. Paper chromatography of a neutralised, acid hydrolysate of the desulphated polymer revealed that the 4-O-methylgalactose spot (spray a) was less intense than the 2-O-methylgalactose spot. Although exact measurements were not possible, GLC studies showed that the ratio of galactose : 4-O-methylgalactose : 2-O-methylgalactose was 35 : 1 : 2.

2.5. Alkali-treatment of the polysaccharide

To the polysaccharide (1.5 g) in water (200 ml) was added sodium borohydride [42] (0.5 g) and the mixture was stored for 48 h at room temperature. Aqueous sodium hydroxide (40%, 50 ml) and sodium borohydride (1.5 g) were then added, and the mixture was heated at 70-75° for 7 h, with additions of sodium borohydride (0.5 g) every hour. This process was repeated in a second experiment, but with the water bath maintained at 80 ± 1°. The solutions were dialysed, the dialysates were centrifuged and concentrated, and the polysaccharides were isolated by freeze-drying (1.1 g in each case) Found: 1st experiment; $[\alpha]_D^{26}$ + 43° (c 0.7); 3,6-anhydrogalactose, 2.7; N, 0.0; SO_4^{2-}, 17.5%; 2nd experiment; $[\alpha]_D^{26}$ + 53° (c 1.3); 3,6-anhydrogalactose, 3.5; SO_4^{2-}, 17.9% .

2.6. Periodate oxidation of polysaccharide and desulphated polysaccharide

To the polysaccharide (19.2 mg) and desulphated polysaccharide (19.6 mg; SO_4^{2-}, 6.2%) in water (5 ml) were added equal volumes of 29.8mM sodium metaperiodate and the oxidations were followed spectrophotometrically [124] (Table III). The solutions were then treated with excess of ethylene glycol, dialysed, and concentrated. Paper chromatography (solvents 1 and 2) of a neutralised, acid hydrolysate showed only galactose and 2-O-methylgalactose in each case. In order to obtain sufficient oxopolysaccharide for further examination, the polysaccharide (387 mg) in water (50 ml) was treated with an equal volume of 59.4mM sodium

metaperiodate, the reduction of periodate being followed titrimetrically [125] (Table III). After 150 h, the solution (now reduced to 35 ml; 65 ml having been used for the titrimetry) was treated with excess of ethylene glycol and dialysed. The oxopolysaccharide (40 mg) was isolated by concentration of the solution to a suitable volume and freeze-drying. Paper chromatography of a neutralised, acid hydrolysate of the oxopolysaccharide indicated galactose and 2-O-methylgalactose, but 4-O-methylgalactose and xylose were not detected. GLC (conditions outlined earlier) showed that the molar ratio of D-galactose to 2-O-methyl-D-galactose in the oxopolysaccharide was 6.1 : 1, a peak corresponding to 4-O-methyl-L-galactose was not detected.

2.7. Methylation of desulphated polysaccharide

To a solution of desulphated polysaccharide (9.0 g; SO_4^{2-}, 6.2%) in methyl sulphoxide (150 ml) was added solid sodium hydroxide (60 g) and methyl sulphate (30 ml) over a period of 6 h with vigorous stirring. The mixture was then stirred overnight. This treatment was repeated, after which the residual methyl sulphate was destroyed by heating on a boiling water-bath for 1.5 h. Water was added to dissolve the solids, and the alkaline solution was partially neutralised in the cold with 2.5M sulphuric acid, the neutralisation being completed with dilute acetic acid. This solution (1.5 l) was dialysed, concentrated to a suitable volume, and freeze-dried to a solid foam (12.6 g). After a second methylation, the methylated polymer was extracted into chloroform in the usual

way, yielding a glassy foam (A) (1.1 g), which had no i.r. band for hydroxyl groups. Paper chromatography (solvents 1 and 5, spray a) of a neutralised, acid hydrolysate showed 2,3,6- and 2,4,6-tri-O-methylgalactoses, with smaller amounts of 2,3,4,6-tetra-O-methylgalactose, some di-O-methylgalactoses, and 2-O-methylgalactose. In view of the difficulty of methylating on a large scale with Purdie's reagents [126], a small quantity of A (35 mg) was exhaustively methylated by stirring in methyl iodide (12 ml) with freshly prepared silver oxide (2 g added in portions over 4 days). The mixture was filtered and concentrated to a glass, and the above methylation procedure was repeated. Finally, the polymer was given two more treatments as above, the silver oxide (3 g each) being added while the solution was under reflux, to yield a glass (B, 24 mg). Paper chromatography (solvents 1 and 5) of a neutralised, acid hydrolysate of B showed all the sugars present in the hydrolysate of A. The infrared spectrum of B showed no hydroxyl peak (Found: OMe, 37.4%).

2.8. Hydrolysis of the methylated, desulphated polysaccharide

The methylated, desulphated polysaccharide (1 g; SO_4^{2-}, 6.2%) described above was hydrolysed by the formic acid - dilute sulphuric acid method [127], to yield a syrup which was applied to a charcoal - Celite (1 : 1) column (45 x 500 mm). The methylated sugars were eluted by applying a linear gradient of 0-5% butanone in water over a volume of 12 l. Fractions (30 ml) were collected, analysed by paper chromatography, and combined into the following fractions.

Fraction I. The syrup contained minute traces of galactose and was discarded.

Fraction II. The syrup (130 mg) was further fractionated by paper chromatography (solvent 5) into 2(a) a syrup (60 mg) chromatographically (solvents 1 and 5, sprays a and d) identical with 2-O-methylgalactose; and 2(b) a syrup (60 mg) which was shown by paper chromatography (solvents 1 and 5, sprays a and d) to be a mixture of di-O-methylgalactoses; this fraction was not further investigated.

Fraction III. The syrup (20 mg) was shown by paper chromatography (solvents 1 and 5) to be 2,4,6-tri-O-methylgalactose containing traces of di-O-methylgalactoses and 2-O-methylgalactose.

Fraction IV. The syrup (147 mg), which was chromatographically identical with 2,4,6-tri-O-methyl-D-galactose, had $[\alpha]_D^{19}$ + 82° (c 0.98) and yielded an "anilide" which, after two recrystallisations from ethanol, had m.p. 171-172° alone and in admixture with 2,4,6-tri-O-methyl-N-phenyl-D-galactosylamine (93).

Fraction V. The syrup (210 mg) was shown by paper chromatography (solvents 1 and 5) to be a mixture of 2,3,6- and 2,4,6-tri-O-methylgalactose.

Fraction VI. The syrup (117 mg) was shown by paper chromatography (solvents 1 and 5) to be 2,3,6-tri-O-methylgalactose containing traces of 2,4,6-tri-O-methylgalactose and 2,3,4,6-tetra-O-methylgalactose. The derived 2,3,6-tri-O-methyl-D-galactonolactone (128), after recrystallisation from dry ether,

had m.p. and mixed m.p. 97-98°.

Fraction VII. The syrup (105 mg), $[\alpha]_D^{20}$ + 74° (c 1.18), was shown by paper chromatography (solvents 1,4 and 5) to be pure 2,3,6-tri-O-methyl-D-galactose.

Fraction VIII. The syrup (68 mg), $[\alpha]_D^{19}$ + 2° (c 3.1), was shown by paper chromatography (solvents 1,4 and 5) to be 2,3,4,6-tetra-O-methylgalactose. The aniline derivative, after several recrystallisations from ethanol, had m.p. 179-180°, which could not be increased by further recrystallisations. The mixed m.p. (with authentic 2,3,4,6-tetra-O-methyl-N-phenyl-D-galactosylamine of m.p. 189-190°) was 181°. This m.p. behaviour of the "anilide", together with the optical rotation of the syrup, suggests that this fraction is a mixture of approximately equal parts of tetra-O-methyl-D- and -L-galactoses.

Fraction IX. The syrup (53 mg) contained degradation products arising from the hydrolysis and was not further investigated.

2.9. Partial hydrolysis of the Polysaccharide

In order to determine the optimum conditions for the formation of oligosaccharides during partial hydrolysis of the native sulphated polysaccharide, three quantities of polysaccharide (1 g each) were heated with 0.25, 0.375 and 0.5M H_2SO_4 on a boiling-water bath. Aliquots (1 ml) were drawn off at regular intervals, neutralized ($BaCO_3$), centrifuged, concentrated and examined by paper chromatography (solvent 1,

spray a) The maximum concentration of oligosaccharides was found after 2 h hydrolysis with 0.375M H_2SO_4. Polysaccharide (20 g) was hydrolysed for 2 h with 0.375M H_2SO_4 (250 ml), neutralized ($BaCO_3$), centrifuged, and evaporated to a syrup. The syrup, after dissolution in water (200 ml), was deionized by passage first through a column (250 x 35 mm) of Amberlite IR - 120 (H^+) resin, the column being washed with distilled water (400 ml) until the eluant was only just positive to Molisch reagent. This solution was then applied to a column (250 x 35 mm) of Amberlite IRA 400 in the acetate form. The column was washed with distilled water (300 ml) until the eluant was free of carbohydrate. The solution thus obtained was evaporated to a neutral syrup (7.5 g). This syrup was applied to a charcoal-Celite column (1 : 1 w/w; 560 x 55 mm), and eluted with water and aqueous ethanol (0 - 16% ethanol in water) using the gradient technique. Fractions (ca. 30 ml) were collected and sorted by paper chromatography into 15 major fractions.

Fraction I. A syrup (2.52 g), eluted with water (3.9 l), was shown by paper chromatography to be predominantly galactose, together with small traces of 4-O-methylgalactose and four fast running saccharides (spray a).

Fraction II. The crystalline solid (190 mg), eluted with water (1.2 l), was shown by paper chromatography to be mostly 4-O-methylgalactose with some galactose.

Fraction III. The half-crystallised syrup (900 mg), eluted with water (4.2 l), contained mostly 4-O-methylgalactose

and 2-O-methylgalactose together with traces of two fast moving saccharides.

Fraction IV. The white solid (136 mg), was eluted with 1% aqueous ethanol (1.2 l) and was shown by paper chromatography (solvents 1 and 5, sprays a and d) to contain 2-O-methylgalactose (major component), 4-O-methylgalactose and 6-O-methylgalactose. This fraction was further fractionated on Whatman No. 1 paper using solvent 5. Extraction of the relevant portion of the papers with hot water : ethanol (1 : 1) yielded a syrup (~ 2 mg) with $[\alpha]_D$ ca. + 30° (c ~ 0.2, murky solution). The chromatographically pure syrup was chromatographically identical with 6-O-methylgalactose (solvents 1 and 5, sprays a and d). The low but positive specific rotation indicates that at least the D-isomer is present.

Fraction V. The white solid (140 mg), eluted with 2% aqueous ethanol (1.3 l), was shown by paper chromatography (solvent 1, spray a) to contain three oligosaccharides with R_{gal} 0.21, 0.33 and 0.36. The mixture was further fractionated on Whatman 3 MM paper using solvent 1.

Oligosaccharide 1. The syrup (16 mg) was chromatographically pure (solvents 1 and 5) and had $[\alpha]_D^{25}$ + 11 (c 0.58), R_{gal} 0.21, D.P. $^{(129)}$ 2.1. The oligosaccharide was revealed as a pink spot when a chromatogram was sprayed with spray d. Complete and partial hydrolysis for this and all further oligosaccharides was performed as follows. Oligosaccharide (1-2 mg) was heated with 0.4 - 0.5M H_2SO_4 (ca. 1 ml) on a boiling water bath (time dependent on degree of hydrolysis required). The hydrolysate was neutralized ($BaCO_3$), centrifuged, deionized on

a small mixed bed resin column (Amberlite IR 120 (H^+) and Amberlite IRA 400 in the acetate form) and evaporated to a syrup under reduced pressure. Examination of the hydrolysate of oligosaccharide 1 by paper chromatography (solvents 1 and 5) revealed the presence of only galactose, while partial hydrolysis showed galactose and starting material. Oligosaccharide 1 was methylated by a modified Hakomori [130,131] procedure as follows. Oligosaccharide (2 mg) was dissolved in dimethyl sulphoxide (1.2 ml) and sodium hydride (40 mg) added to the solution which was then ultrasonicated for 30 min under nitrogen. The cloudy solution was cooled in ice, and methyl iodide (0.6ml) was added slowly. Ultrasonication was continued for a further 20 min under nitrogen, when a clear light yellow solution was obtained. Chloroform (5 ml) was added and the solution extracted twice with water (2 ml). The chloroform fraction was dried (Na_2SO_4) and evaporated to a yellow syrup. Hydrolysis of the syrup (0.5M H_2SO_4, 2 ml) followed by neutralization ($BaCO_3$), deionization on a small mixed bed resin column (Amberlite IR 120 (H^+) and Amberlite IRA 400 in the acetate form) and evaporation to dryness under reduced pressure afforded a clear syrup. Examination of the hydrolysate by paper chromatography and TLC revealed spots with the mobilities of 2,3,4,6-tetra-O-methylgalactose, 2,3,4-tri-O-methylgalactose and some degraded material (the thin-layer chromatographic pattern for this hydrolysate was identical with that of a similar hydrolysate obtained from methylated oligosaccharide 4). The hydrolysate was methanolysed by refluxing with 1M methanolic hydrogen chloride (3 ml) for 4 h, followed by neutralization (Ag_2CO_3), centrifugation and concentration. Examination of the methylglycosides by GLC showed peaks corresponding to methyl 2,3,4,6-tetra-O-

methylgalactoside (T 1.58), methyl 2,3,4-tri-O-methylgalactoside (T 5.81) and the degradation peak with T 3.71. Oligosaccharide 1 is thus 6-O-β-D-galactopyranosyl-D-galactose, the β-configuration being assumed from the optical rotation. (The hydrolysate of the oligosaccharide had a positive rotation indicating only D-galactose).

The oligosaccharide R_{gal} 0.33 was chromatographically identical with 4-O-β-D-galactopyranosyl-L-galactose, but it was only present in trace quantity and was lost on the Whatman 3 MM paper. The oligosaccharide with R_{gal} 0.36 was chromatographically identical with oligosaccharide 2.

Fraction VI. The semi-crystalline syrup (780 mg), was eluted with 3% ethanol. Paper chromatography showed that the fraction consisted mainly of oligosaccharide 2 with traces of oligosaccharide 4.

Oligosaccharide 2. The saccharide crystallized readily from aqueous methanol, and after recrystallization had $[\alpha]_D^{20}$ + 87° (5 min)→ + 64° (c 0.66), m.p. (decomposition) 203 - 204°, R_{gal} 0.36. Mixed m.p. with authentic 4-O-β-D-galactopyranosyl-D-galactose of m.p. 196° was 199 - 201°. Complete hydrolysis followed by paper chromatography showed galactose only, while partial hydrolysis showed galactose and the original material. The oligosaccharide was methylated by the modified Hakomori method [130,131] outlined for oligosaccharide 1. Examination of the hydrolysate of the methylated oligosaccharide by paper chromatography (spray a) and TLC (sprays b and c) showed only two spots with the chromatographic mobilities of 2,3,4,6-tetra-O-methylgalactose and 2,3,6-tri-O-methylgalactose. Examination of a methanolysate of the hydrolysed methylated

oligosaccharide by GLC showed peaks corresponding to methyl 2,3,4,6-tetra-O-methylgalactoside (T 1.59) and methyl 2,3,6-tri-O-methylgalactoside (T 2.74, 3.27, 3.58 and 3.86). The infrared spectrum of this oligosaccharide was identical with that of authentic 4-O-β-D-galactopyranosyl-D-galactose.

Fraction VII. The syrup (250 mg), eluted with 5% ethanol, was shown by paper chromatography to consist of a mixture of mainly oligosaccharides 2 and 4 with a trace of oligosaccharide 5. It was further fractionated on Whatman 3 MM paper using solvent a.

Oligosaccharide 4. The syrup (50 mg) was obtained by extraction of the relevant portions of the papers with hot aqueous methanol. It was chromatographically pure in solvents 1 and 2 (spray a), and had $[\alpha]_D^{25}$ - 69° (c 1.18), R_{gal} 0.47. It gave a strong pink spot with spray d, indicating that the oligosaccharide is not 1,2-linked. Hydrolysis of the oligosaccharide gave galactose and 4-O-methylgalactose (paper chromatography, solvents 1 and 2) while partial hydrolysis afforded galactose, 4-O-methylgalactose and the original material. Oligosaccharide (2 mg) in water (0.5 ml) was reduced with sodium borohydride (20 mg) for 1½ h. The reduced saccharide was hydrolysed with 0.5M H_2SO_4 (5 ml) for 2 h, neutralized ($BaCO_3$) and evaporated to dryness under reduced pressure (boric acid removed by codistillation with methanol). Paper chromatography (solvent 1) showed only 4-O-methylgalactose. The oligosaccharide was methylated by a modified Hakomori [130, 131] procedure (see oligosaccharide 1). Examination of a hydrolysate of the methylated oligosaccharide

by TLC (sprays b and c) showed spots corresponding to 2,3,4,6-tetra-O-methylgalactose and 2,3,4-tri-O-methylgalactose with some degradation product, while paper chromatography (solvent 5) showed 3 spots with spray a corresponding to those observed on the TLC; when a similar paper was sprayed with spray d only a faint pink spot due to the degradation product was observed. The hydrolysate was refluxed with 1M methanolic hydrogen chloride for 4 h, neutralized (Ag_2CO_3), deionized and concentrated. GLC of the methanolysate showed three peaks with retention times corresponding to methyl 2,3,4,6-tetra-O-methylgalactoside (T 1.60), methyl 2,3,4-tri-O-methylgalactoside (T 5.85) and the unidentified peak at T 3.76 (cf. oligosaccharide 1). Oligosaccharide 4 is thus 6-O-α-(4-O-methyl-L-galactopyranosyl)-D-galactose. The α-configuration is assumed from the optical rotation.

Fraction VIII. The syrup (246 mg), eluted with 5 - 10½% aqueous ethanol, was shown by paper chromatography to be a mixture of oligosaccharides 2,4,5,6 and 7, and it was further fractionated on Whatman No. 1 paper using solvent 1.

Oligosaccharide 5. The syrup (12 mg) was extracted from the relevant portions of paper using hot aqueous methanol, and was chromatographically pure (solvents 1 and 5). It had $[\alpha]_D^{25}$ + 100° (c 0.40), R_{gal} 0.61. Examination of a hydrolysate by paper chromatography showed galactose and 2-O-methylgalactose. Reduction of the oligosaccharide with sodium borohydride followed by hydrolysis of the reduced saccharide showed only 2-O-methylgalactose (paper chromatography, solvent 1). The oligosaccharide is chromatographically identical (solvents 1

and 5) with oligosaccharide 5 obtained from the acetolysis of the polysaccharide.

Fraction IX. The syrup (270 mg), eluted with 10½% aqueous ethanol, was shown by paper chromatography to be almost pure oligosaccharide 7. The syrup crystallized on trituration with methanol and after recrystallization had m.p. and mixed m.p. 211 - 214°, R_{gal} 0.89 (lit. m.p. 213 - 214°) for authentic 4-O-β-D-galactopyranosyl-2-O-methyl-D-galactose). The oligosaccharide failed to give a pink spot with spray d. Paper chromatographic examination (solvents 1 and 3) of a hydrolysate of the oligosaccharide showed galactose and 2-O-methylgalactose, while a partial hydrolysate revealed galactose, 2-O-methylgalactose and the original material. Reduction of the oligosaccharide followed by paper chromatographic examination of its hydrolysate showed galactose but no trace of 2-O-methylgalactose. The infrared spectrum of the oligosaccharide was identical to that of authentic 4-O-β-D-galactopyranosyl-2-O-methyl-D-galactose.

Fraction X. The syrup (95 mg), eluted with 15% aqueous ethanol, was shown by paper chromatography to consist of two slow moving oligosaccharides, and further fractionation was achieved on Whatman No. 1 paper using solvent 1. Neither oligosaccharide was obtained pure, but paper chromatographic examination of hydrolysates of each sub-fraction showed only galactose (solvent 1). The chromatographic mobilities of these two oligosaccharides (solvent 6) are the same as those of oligosaccharides 9 and 10 obtained from the acetolysis of the polysaccharide.

Fraction XI. The syrup (180 mg), eluted with 16% aqueous ethanol, was shown by paper chromatography to consist of a number of oligosaccharides (traces) with one oligosaccharide in a relatively higher concentration. The mixture was further fractionated on Whatman No. 1 paper using solvent 1.

Oligosaccharide 13. The syrup (39 mg) was obtained by extraction of the relevant portions of the papers with hot aqueous methanol. It was chromatographically pure (solvent 1) and had $[\alpha]_D^{21}$ - 32° (c 0.41), R_{gal} 0.15, R_{gg} 0.55. Paper chromatographic examination (solvents 1, 2 and 6) of a hydrolysate showed galactose and 4-O-methylgalactose, while partial hydrolysis showed original material, galactose, 4-O-methylgalactose and oligosaccharides 2 and 4. Oligosaccharide (duplicate experiment) was reduced with sodium borohydride and hydrolysed with 0.25M H_2SO_4 for 7, 30, 75 and 180 min at 100°. Examination of the hydrolysate by paper chromatography showed galactose and 4-O-methylgalactose but no trace of either of the disaccharides 2 and 4 (both of which are relatively stable to acid hydrolysis). Methylation of the oligosaccharide by the modified Hakomori procedure (130, 131), followed by hydrolysis, methanolysation and examination of the methyl glycosides by GLC, showed only one large peak with retention time corresponding to methyl 2,3,4,6-tetra-O-methylgalactosides (T 1.60). The only other peaks present were very much smaller and had relative retention times of T 2.00, 2.80, 3.22, 3.67 and 3.95. No peak was present at or near T 8.38. The oligosaccharide is assigned the structure O-α-(4-O-methyl-L-galactopyranosyl)-(1→6)-O-[β-D-galactopyranosyl-(1→4)]-D-galactose.

Fraction XII. The syrup (100 mg), eluted with 16% aqueous ethanol, was shown by paper chromatography to be a mixture of slow moving oligosaccharides and one very fast moving oligosaccharide (R_{gal} 1.85). The mixture was further fractionated on Whatman No. 1 paper using solvent 1.

Oligosaccharide 8. The syrup (35 mg) was obtained from the relevant portions of the papers by extraction with hot aqueous methanol. It was chromatographically pure in solvents 1 and 5 and had $[\alpha]_D^{22}$ + 15° (c 0.83), R_{gal} 1.85. Complete hydrolysis followed by paper chromatography revealed 6-O-methylgalactose and 2-O-methylgalactose (solvents 1 and 2), while partial hydrolysis afforded 6-O-methylgalactose, 2-O-methylgalactose and the original material. Oligosaccharide was reduced with sodium borohydride : hydrolysis of the non-reducing syrup, followed by paper chromatography (solvent 2), revealed 6-O-methylgalactose and a very small trace of galactose (demethylation product), but no trace of 2-O-methylgalactose. The oligosaccharide was methylated by the method of Haq and Percival [132]. Oligosaccharide (1 mg) was dissolved in redistilled N,N-dimethylformamide (0.3 ml) and redistilled methyl iodide (0.3 ml) and dry freshly prepared silver oxide (0.25 g) added at 0°. The mixture was shaken in a sealed ampoule in the dark for 6 h. The completely methylated oligosaccharide was extracted with chloroform (3 ml) and the solution evaporated to dryness. Complete acid hydrolysis of the permethylated oligosaccharide followed by paper chromatography (solvent 5, spray a) showed spots corresponding to 2,3,4,6-tetra-O-methylgalactose and 2,3,6-tri-O-methylgalactose. GLC of the methanolysed hydrolysate revealed

peaks with retention times corresponding to methyl 2,3,4,6-tetra-O-methylgalactoside (T 1.63) and methyl 2,3,6-tri-O-methylgalactoside (T 2.78, 3.34, 3.64 and 3.91), the two saccharides being in a 1 : 1 molar ratio. The oligosaccharide is thus assigned the structure 4-O-β-(6-O-methyl-D-galactopyranosyl)-2-O-methyl-D-galactose. The β-configuration is assumed from the oligosaccharide's low positive optical rotation.

Fraction XIII. The rest of the material (500 mg), eluted with up to 50% aqueous ethanol was a mixture of oligosaccharides with very low chromatographic mobility, and was not further investigated.

2.10. Acetolysis of Polysaccharide

Freeze-dried polysaccharide (30 g) was added to a mixture of acetic anhydride (160 ml), acetic acid (120 ml) and sulphuric acid (16 ml) with continuous stirring over a period of 10 min. The mixture was shaken for 100 h at room temperature, and then decanted from the precipitate. The clear solution was mixed with ice-cold water (1.6 l) and neutralized with sodium bicarbonate until neutral to congo red (ca. pH 5). The solution was filtered and the precipitate dissolved in chloroform. The clear filtrate was extracted with chloroform (each 400 ml aqueous solution extracted with 3 x 100 ml chloroform). The combined chloroform solutions were dried with sodium sulphate and evaporated to a syrup, which was dissolved in water and freeze-dried (19.4 g).

The acetylated oligosaccharides were deacetylated by the addition of sodium methoxide (1.0 g sodium in 200 ml methanol) to a solution of the saccharides in methanol (200 ml) with vigorous stirring, which was continued for 5 h at room temperature. The stoichiometric quantity of acetic acid in water (400 ml) was added to neutralize the sodium hydroxide. Deionization of the solution was effected by passing the solution first through an Amberlite IR - 120 (H^+) column (34 x 670 mm) and then through a column (34 x 480 mm) of Amberlite IRA 400 in the acetate form. The neutral eluant was evaporated to a syrup and the oligosaccharides isolated by freeze-drying (9.2 g).

The oligosaccharide mixture was applied to a charcoal-celite column (51 x 730 mm; 1 : 1 w/w) and eluted with water (15 l) and aqueous ethanol (74 l of 0 - 17% ethanol in water) using the gradient technique. Fractions (ca. 450 ml) were collected and sorted by paper chromatography into 13 major fractions. The column was finally washed with 40% ethanol (34 l) and then with 10% butanone until essentially free of carbohydrate.

Fractions I to III. The white solids (1.94, 0.58 and 1.21 g respectively), eluted with water and 1.5% aqueous ethanol (26.5 l), were shown by paper chromatography to be predominantly galactose, with smaller quantities of 4-O-methylgalactose and 2-O-methylgalactose and traces of four saccharides with $R_{gal} > 1$. The fractions were not further investigated.

Fraction IV. The syrup (67 mg), eluted with 1.5% aqueous ethanol (1.7 l), was shown by paper chromatography to be a mixture of mostly 4-O-methylgalactose and 2-O-methylgalactose with traces of galactose, 6-O-methylgalactose and an oligosaccharide with R_{gal} 0.3.

Fraction V. The syrup (1.45 g), eluted with 7% aqueous ethanol (15.7 l) was shown by paper chromatography to be a mixture of mono- and oligo-saccharides. Half of this fraction was fractionated on Whatman No. 1 paper using solvent 5.

Oligosaccharide 3. Extraction of the relevant portions of the papers with hot aqueous methanol yielded a syrup (440 mg) which appeared to be more than 95% chromatographically pure in solvents 1, 5 and 6. It had $[\alpha]_D^{25}$ + 124° and R_{gal} 0.35. Complete hydrolysis of the oligosaccharide (see oligosaccharide 1 from partial hydrolysis of polysaccharide) gave only galactose, while paper chromatography of a partial hydrolysate gave galactose and the original material. The oligosaccharide was methylated by the modified Hakomori method [130, 131] outlined for oligosaccharide 1. Examination of the hydrolysate of the methylated oligosaccharide by paper chromatography (spray a) and TLC (sprays b and c) showed two spots with the chromatographic mobilities of 2,3,4,6-tetra-O-methylgalactose and 2,4,6-tri-O-methylgalactose. Gas chromatographic examination of a methanolysate of the hydrolysed methylated oligosaccharide showed peaks corresponding to methyl 2,3,4,6-tetra-O-methylgalactosides (T 1.60), methyl 2,4,6-tri-O-methylgalactosides (T 3.55, 3.92) and the peak at T 3.72 (due to the product of partial alkali degradation of the 1,3-linkage [99]). The presence of a small peak at T 2.78

is ascribed to a small amount of contaminating oligosaccharide with 1,4-linkage, since T 2.78 is the first and largest peak of methyl 2,3,6-tri-O-methylgalactosides. The oligosaccharide is thus assigned the structure 3-O-α-D-galactopyranosyl-D-galactose. The α-configuration follows from the specific rotation.

Fraction VI. The syrup (175 mg), eluted with 8% aqueous ethanol (3.6 l), was shown by paper chromatography to be a mixture of mainly oligosaccharides 3,5,6 with small traces of three saccharides with $R_{gal} \geq 1$. It was further fractionated on Whatman No. 1 paper using solvent 5.

Oligosaccharide 5. The syrup (31 mg), obtained by extraction of the relevant portions of the papers with hot aqueous methanol was chromatographically pure in solvents 1 and 5 and had R_{gal} 0.61, $[\alpha]_D^{25}$ + 130°. The oligosaccharide gave a pink spot with spray d. Paper chromatographic examination of a neutralized hydrolysate showed galactose and 2-O-methylgalactose, while partial hydrolysis revealed galactose, 2-O-methylgalactose and starting material. Reduction of the oligosaccharide with sodium borohydride followed by hydrolysis and paper chromatography revealed only 2-O-methylgalactose. Methylation of the oligosaccharide by two methods [130,131, 132] followed by examination of the methanolysate by gas chromatography showed peaks corresponding to methyl 2,3,4,6-tetra-O-methylgalactosides (T 1.60) and the alkali degradation peak at T 3.75. There were only slight shoulders on this peak corresponding to the twin peaks of the methyl 2,4,6-tri-O-methylgalactosides. However, this oligosaccharide is chromatographically identical with one of the partial hydrolysis

products of oligosaccharide 12, where the linkage has been established as 1,3 by methylation of the trisaccharide. Oligosaccharide 5 is thus assigned the structure 3-O-α-(2-O-methyl-D-galactopyranosyl)-D-galactose. The α-configuration is assumed from the optical rotation.

Oligosaccharide 6. The syrup (24 mg), obtained by extraction (hot aqueous methanol) of the upper half of what appeared to be a single saccharide band on the papers, was chromatographically pure in solvents 1 and 5 and had $[\alpha]_D^{25}$ - 33°, R_{gal} 0.74. Hydrolysis of the oligosaccharide followed by paper chromatography revealed galactose and 2-O-methylgalactose, while hydrolysis of the reduced oligosaccharide showed only galactose. The oligosaccharide failed to give a pink spot with spray d. Separation of the hydrolysis products of the oligosaccharide by paper chromatography (solvent 1) gave D-galactose $[\alpha]_D^{20}$ + 47° (c 0.131) and 2-O-methyl-L-galactose $[\alpha]_D^{20}$ - 44° (c 0.133). The oligosaccharide was methylated by a modified Hakomori method [130,131], followed by hydrolysis and TLC and paper chromatographic examination. Spots corresponding to 2,3,4,6-tetra-O-methylgalactose and 2,3,6-tri-O-methylgalactose were observed. Examination of a methanolysate of this hydrolysate by GLC showed peaks with the relative retention times of methyl 2,3,4,6-tetra-O-methylgalactosides (T 1.61) and methyl 2,3,6-tri-O-methyl-galactoside (T 2.79, 3.38, 3.67 and 3.96). Oligosaccharide 6 is thus assigned the structure 4-O-β-D-galactopyranosyl-2-O-methyl-L-galactose.

The lower half of the saccharide band (the top half of which had yielded oligosaccharide 6) gave a syrup (12 mg)

which appeared to be almost chromatographically pure (solvents 1 and 5) and had $[\alpha]_D^{25}$ + 28°, R_{gal} 0.74. Paper chromatographic examination of a hydrolysate showed galactose and 2-O-methylgalactose while a hydrolysate of the reduced oligosaccharide showed only galactose. Separation of the hydrolysate by paper chromatography gave D-galactose $[\alpha]_D^{20}$ + 44° (c 0.095) and 2-O-methylgalactose $[\alpha]_D^{20}$ - 7° (c 0.106) showing that the 2-O-methylgalactose is a mixture of the D- and L-isomers. Methylation of the original syrup and examination of a methanolysate by GLC showed peaks corresponding to methyl 2,3,4,6-tetra-O-methylgalactosides (T 1.63) and methyl 2,3,6-tri-O-methylgalactosides (T 2.83 3.42, 3.66 and 3.99). These results indicate that the syrup is a mixture of oligosaccharides 6 and 7.

Fraction VII. The syrup (182 mg), eluted with 9% aqueous ethanol (3.6 l), was shown by paper chromatography to be a mixture of mainly three saccharides with R_{gal} 0.81, 1.16 and 1.43 (solvent 5) with traces of oligosaccharides 3, 5 and 6 and oligosaccharides with $R_{gal} < 0.3$. The mixture was further fractionated on Whatman No. 1 paper using solvent 5.

Extraction of the portions of the papers at R_{gal} 0.89 (solvent 5) yielded a chromatographically pure syrup (45 mg) with $[\alpha]_D^{25}$ + 57°, R_{gal} 0.89. The chromatographic mobility was identical with that of oligosaccharide 7 in several solvent systems. Hydrolysis of the syrup, followed by paper chromatography revealed galactose and 2-O-methylgalactose, while hydrolysis of the reduced oligosaccharide gave galactose only. All these facts indicate that the sugar is identical

with oligosaccharide 7, isolated from the partial hydrolysis of the polysaccharide i.e. 4-O-β-D-galactopyranosyl-2-O-methyl-D-galactose.

Extraction of the relevant portions of the papers at R_{gal} 1.16 (solvent 5) yielded a chromatographically pure syrup (9 mg) with $[\alpha]_D^{25}$ + 110°. Hydrolysis of the syrup followed by paper chromatography revealed only galactose. Methylation of the syrup by the modified Hakomori method (130,131), followed by hydrolysis, methanolysis and GLC examination, showed a large peak at T 1.61 (methyl 2,3,4,6-tetra-O-methylgalactosides) with much smaller peaks at T 2.01, 2.47, 2.80, 3.22 and 3.95 (cf. oligosaccharide 13). The saccharide was not further investigated.

The third saccharide at R_{gal} 1.43 (solvent 5) was obtained as a chromatographically pure syrup (4 mg) by extraction of the relevant portions of the papers with hot aqueous methanol. It had $[\alpha]_D^{25}$ + 4°, was completely degraded on acid hydrolysis (paper chromatography), and was not further investigated.

Fraction VIII. The syrup (718 mg), eluted with 11% aqueous ethanol (7.5 l), was shown by paper chromatography to be a mixture of three oligosaccharides with R_{gg} 0.44, 0.51 and 0.64. The mixture was separated on Whatman No. 1 paper using solvent 6 to give oligosaccharides 9 and 10.

Oligosaccharide 9. The syrup (133 mg), obtained by extraction of the relevant portions of the papers with hot aqueous methanol, was chromatographically pure in solvents 1 and 6, and had $[\alpha]_D^{25}$ + 103°, R_{gg} 0.51. Paper chromatographic examination of a hydrolysate of the oligosaccharide showed only galactose, while

partial hydrolysis gave galactose, oligosaccharide 2, oligosaccharide 3 and starting material. Reduction of the oligosaccharide ($NaBH_4$) followed by partial hydrolysis and paper chromatography revealed galactose and oligosaccharide 2, but no trace of oligosaccharide 3. Methylation of the oligosaccharide by a modified Hakomori procedure (130,131) (cf. oligosaccharide 1), followed by hydrolysis and paper chromatography showed spots corresponding to 2,3,4,6-tetra-O-methylgalactose, 2,3,6-tri-O-methylgalactose and 2,4,6-tri-O-methylgalactose. When the hydrolysate was methanolysed and examined by gas chromatography, it showed peaks corresponding to methyl 2,3,4,6-tetra-O-methylgalactosides (T 1.63), methyl 2,3,6-tri-O-methylgalactosides (T 2.78, (3.42), (3.66) and (3.93)). methyl 2,4,6-tri-O-methylgalactosides (T (3.95) and 3.93) and the peak at T 3.74 (degradation product). Oligosaccharide 9 is thus assigned the structure O-β-D-galactopyranosyl-(1→4)-O-α-D-galactopyranosyl-(1→3)-D-galactose.

Oligosaccharide 10. Extraction of the relevant portions of the above papers with hot aqueous methanol afforded a chromatographically pure syrup (290 mg) which crystallized readily, and on recrystallization from aqueous ethanol had m.p. 229-232°, $[\alpha]_D^{25}$ + 125°, R_{gg} 0.64. Examination of a hydrolysate of the oligosaccharide by paper chromatography revealed galactose, while a partial hydrolysate revealed galactose, oligosaccharides 2 and 3 and starting material. Reduction of the oligosaccharide ($NaBH_4$) followed by partial hydrolysis and paper chromatography, showed galactose and oligosaccharide 3, but no trace of oligosaccharide 2. Methylation of the oligosaccharide by a modified Hakomori

procedure [130,131] followed by hydrolysis and paper chromatography showed spots corresponding to 2,3,4,6-tetra-O-methylgalactose, 2,3,6 -tri-O-methylgalactose and 2,4,6-tri-O-methylgalactose. When the hydrolysate was methanolysed and examined by gas chromatography it showed peaks corresponding to methyl 2,3,4,6-tetra-O-methylgalactosides (T1.64), methyl 2,3,6-tri-O-methylgalactosides (T 2.79, (3.42), (3.66) and (3.95)) and methyl 2,4,6-tri-O-methylgalactosides (T 3.57 and 3.96). Oligosaccharide 10 is thus assigned the structure O-α-D-galactopyranosyl-(1→3)-O-β-D-galactopyranosyl-(1→4)-D-galactose.

Fraction IX. The syrup (98 mg), eluted with 12% aqueous ethanol (5.0 l), was shown by paper chromatography to be a mixture of oligosaccharides with R_{gg} 0.44, 0.51, 0.64 and 0.88, the latter in only a trace amount. The mixture was not further investigated.

Fraction X. The syrup (426 mg), eluted with 14% aqueous ethanol (9.0 l), was shown by paper chromatography to be a mixture of slow moving oligosaccharides chromatographically identical with those from fraction XI, together with an oligosaccharide with R_{gg} 1.06. The mixture was fractionated on Whatman No. 1 paper using solvent 6.

Oligosaccharide 11. Extraction of the relevant portions of the papers with hot aqueous methanol afforded a syrup (17 mg) which was essentially chromatographically pure in solvents 5 and 6 and had $[\alpha]_D^{25}$ + 126°, R_{gg} 1.06. Hydrolysis of the oligosaccharide followed by paper chromatography revealed galactose and 2-O-methylgalactose, while reduction of the

oligosaccharide followed by hydrolysis and paper chromatography showed only galactose. Paper chromatographic examination of a partial hydrolysate of the oligosaccharide showed galactose, 2-O-methylgalactose, oligosaccharides 3 and 7 and starting material. Methylation of the oligosaccharide by the modified Hakomori procedure [130,131], followed by hydrolysis, methanolysis and gas chromatographic examination of the derived glycosides, showed peaks corresponding to methyl 2,3,4,6-tetra-O-methylgalactosides (T 1.59), methyl 2,4,6-tri-O-methylgalactosides (T 3.51 and 3.91) and methyl 2,3,6-tri-O-methylgalactosides (T 2.76, (3.42), (3.66) and (3.95)). Oligosaccharide 11 is thus assigned the structure O-α-D-galactopyranosyl-(1→3)-O-β-D-galactopyranosyl-(1→4)-2-O-methyl-D-galactose.

Fraction XI. The syrup (445 mg), eluted with 15% aqueous ethanol (6.5 l), was shown by paper chromatography to be a mixture of oligosaccharides with R_{gg} 0.19, 0.25, 0.29 and 0.41. The mixture was fractionated on Whatman No. 1 paper using solvent 6.

Oligosaccharide 14. Extraction of the relevant portions of the papers with hot aqueous methanol afforded a syrup (8 mg) which was chromatographically pure in solvents 1 and 6 and had $[\alpha]_D^{25}$ - 60°, R_{gg} 0.19. Hydrolysis of the oligosaccharide (0.5M H_2SO_4) followed by neutralization ($BaCO_3$), centrifugation, deionization on a mixed bed of Amberlite IR 120 (H^+) and Amberlite IRA 400 (CH_3COO^-) resins, and evaporation under reduced pressure afforded a clear syrup, which was shown by paper chromatography to contain only galactose. Measurement

of the optical rotation of the syrup gave $[\alpha]_D^{25}$ + 5° ± 3° (c 0.057) indicating that the galactose is present as a racemate. Partial hydrolysis of the oligosaccharide followed by neutralisation ($BaCO_3$), centrifugation, deionization and paper chromatography showed two trisaccharides (the faster one with the mobility of O-β-D-galactopyranosyl-(1→4)-O-α-L-galactopyranosyl-(1→3)-D-galactose) and one disaccharide spot with the same mobility as 4-O-β-D-galactopyranosyl-L-galactose and 3-O-α-L-galactopyranosyl-D-galactose (which have the same mobility in solvent 6). No saccharides with the mobilities of oligosaccharides 2 or 3 were observed, indicating that there are no contiguous D-galactose links in the oligosaccharide which can therefore have only one of three structures viz. : D-L-D-L, L-D-L-D or D-L-L-D where L and D are L- and D-galactose respectively. Since a spot with the mobility of the D-L-D trisaccharide was observed in the partial hydrolysis, it is likely that the structure is represented by one of the first two alternatives. An oligosaccharide O-β-D-galactopyranosyl-(1→4)-O-α-L-galactopyranosyl-(1→3)-O-D-galactopyranosyl-(1→4)-L-galactose from Anathecа dentata [101] had $[\alpha]_D$ - 66°.

Oligosaccharide 15. This oligosaccharide was obtained as a chromatographically pure syrup (190 mg) by extraction of the relevant portions of the papers mentioned above. It had $[\alpha]_D^{25}$ + 153° and R_{gg} 0.29. Hydrolysis of the oligosaccharide, followed by neutralization, deionization, concentration and paper chromatography revealed galactose only, while a partial hydrolysate on similar treatment revealed galactose, oligosaccharides 2, 3, 9 and 10 (i.e.

β-$G_D(1\rightarrow4)$-G_D, α-$G_D(1\rightarrow3)$-G_D, β-$G_D(1\rightarrow4)$-α-$G_D(1\rightarrow3)$-G_D and α-$G_D(1\rightarrow3)$-β-$G_D(1\rightarrow4)$-G_D respectively, where G = galactose) and starting material. The oligosaccharide was reduced with sodium borohydride, followed by hydrolysis (0.5M H_2SO_4; 10 min), neutralization ($BaCO_3$), deionization (mixed bed resin), concentration and evaporation of the boric acid with methanol. Paper chromatography of the clear syrup revealed galactose and oligosaccharides 2, 3 and 10, but no trace of oligosaccharide 9. Thus the tetrasaccharide is assigned the structure O-α-D-galactopyranosyl-(1→3)-O-β-D-galactopyranosyl-(1→4)-O-α-D-galactopyranosyl-(1→3)-D-galactose (i.e. α-$G_D(1\rightarrow3)$-β-$G_D(1\rightarrow4)$-α-$G_D(1\rightarrow3)$-G_D where G = galactose).

Fraction XII. The syrup (227 mg), eluted with 17% aqueous ethanol (9.9 l), was shown by paper chromatography to be a mixture of oligosaccharides with R_{gg} 0.19, 0.25, 0.29, 0.41, ca. 1.0 and 1.49. The mixture was separated on Whatman No. 1 paper using solvent 5.

Oligosaccharide 12. The oligosaccharide was obtained as a chromatographically pure syrup (38 mg) by extraction of the relevant portions of the papers with hot aqueous methanol. It had $[\alpha]_D^{25}$ + 109° and R_{gg} 1.49. Hydrolysis of the oligosaccharide followed by paper chromatography revealed galactose and 2-O-methylgalactose, the latter spot appearing twice as intense as the former (spray a). Reduction of the oligosaccharide followed by hydrolysis and paper chromatography revealed galactose and 2-O-methylgalactose, the spots now being of almost equal intensity. The oligosaccharide failed to give a pink spot with spray d. Partial hydrolysis of the oligosaccharide followed by paper chromatography revealed

galactose, 2-O-methylgalactose, oligosaccharides 5 and 7 (i.e. α-2_D-(1→3)-G_D and β-G_D(1→4)-2_D respectively, where G = galactose and 2 = 2-O-methylgalactose) and starting material. Reduction of the oligosaccharide ($NaBH_4$) followed by hydrolysis and paper chromatography revealed galactose, 2-O-methylgalactose and oligosaccharide 5, but no trace of oligosaccharide 7. Methylation of the oligosaccharide by the modified Hakomori procedure [130,131] followed by hydrolysis, methanolysation and gas chromatographic examination of the methyl glycosides, showed peaks corresponding to methyl 2,3,4,6-tetra-O-methylgalactosides (T 1.59), methyl 2,4,6-tri-O-methylgalactosides (T 3.52 and 3.92) and methyl 2,3,6-tri-O-methylgalactosides (T 2.76, (3.42), (3.66) and (3.95)). Reduction of the oligosaccharide, followed by methylation by the modified Hakomori procedure [130,131], hydrolysis, methanolysis and gas chromatographic examination of the derived methyl glycosides showed peaks corresponding to methyl 2,3,4,6-tetra-O-methylgalactosides (T 1.62), methyl 2,4,6-tri-O-methylgalactosides (T 3.51 and 3.93) and a peak at T 2.38 which is considered to have arisen from the permethylated alditol. This establishes the 1,3-linkage on the non-reducing end of the trisaccharide, which in turn establishes the linkage of oligosaccharide 5. Oligosaccharide 12 is thus assigned the structure O-α-(2-O-methyl-D-galactopyranosyl)-(1→3)-O-β-D-galactopyranosyl-(1→4)-2-O-methyl-D-galactose.

Fraction XIII. The syrup (1.56 g) was eluted from the column with 17 - 40% ethanol in water (34 l) using the gradient technique, followed by elution with 10% butanone in water until

the column was essentially free of carbohydrate. The fraction contained high molecular weight material and was not further investigated.

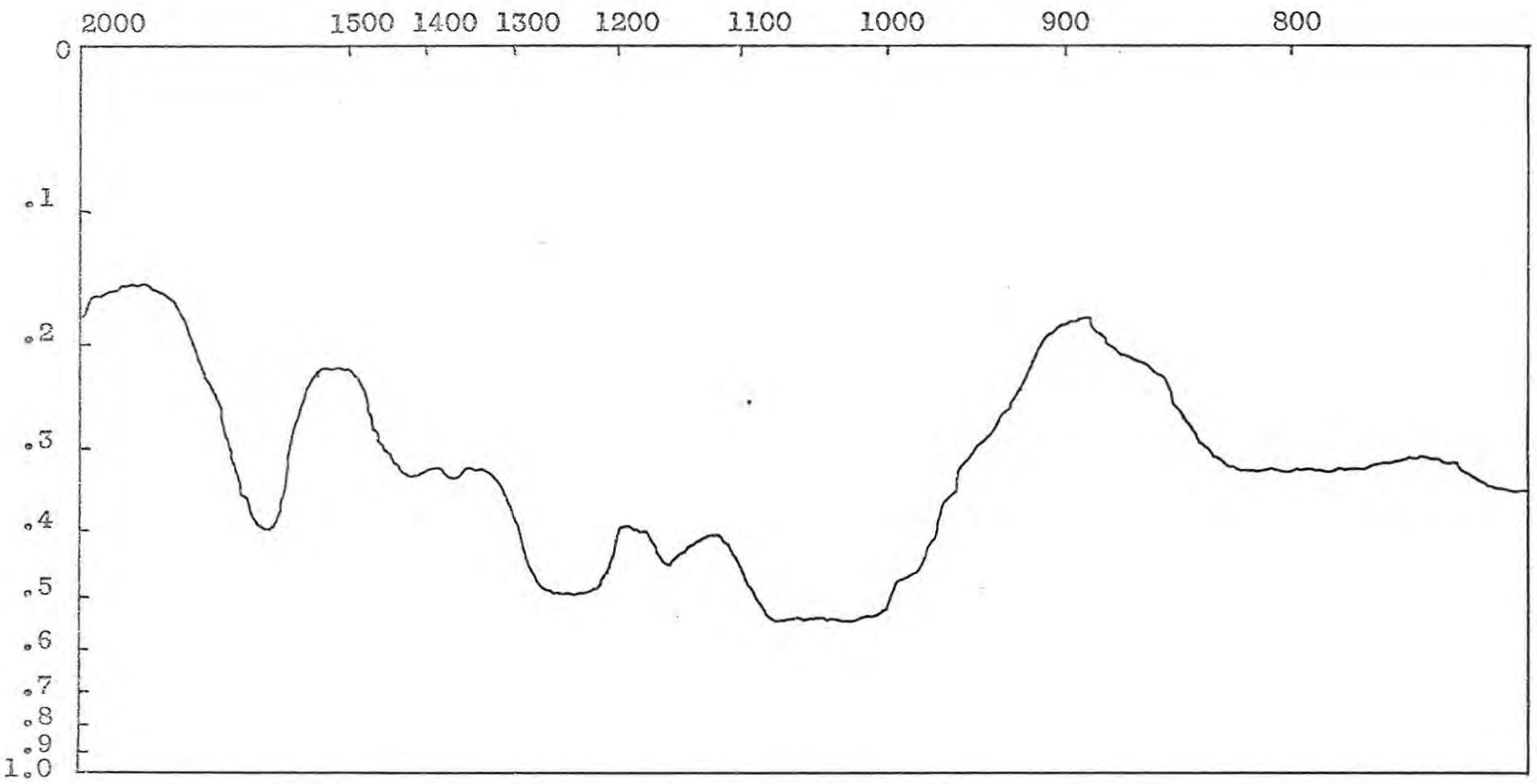
3. DISCUSSION

Aeodes ulvoidea (Schmitz), a red seaweed belonging to the Grateloupiaceae family, was found growing in the intertidal range at Palmiet (ca. 130 miles east of Port Elizabeth). The large, broad, flat fronds are greenish-red in colour, and were harvested in January 1967.

Hot water extraction of the fresh weed, followed by centrifugation and precipitation of the mucilage into ethanol, afforded a sulphated polysaccharide which was contaminated with nitrogenous material. The crude polysaccharide was purified by dissolution in water and centrifugation of the solution, followed by precipitation into ethanol. After several such cycles the thoroughly dried polysaccharide was subjected to ultracentrifuge examination and showed a large, sharp peak, indicating that it is probably an extended type of molecule; in addition the presence of a very small peak showed that the polmer was not completely pure. The polymer had $[\alpha]_D^{23}$ + 34°, failed to precipitate in potassium chloride solution, and gave a faint positive Seliwanoff reaction. The infrared spectrum of the polymer (Figure II) showed the characteristic sulphate ester absorption band at 1240 cm^{-1}, but only a broad shoulder in the 800 - 850 cm^{-1} region, thus giving no indication of the type of the sulphate ester groups present (73, 120).

Fractionation of a neutralised, acid hydrolysate of the polysaccharide, by cellulose-column chromatography, resulted in the isolation of crystalline D-galactose, 4-O-methyl-L-galactose and 2-O-methyl-D-galactose. These saccharides were characterised by their optical rotations, melting points and

FIGURE II : INFRARED SPECTRUM OF NATIVE SULPHATED POLYSACCHARIDE



mixed melting points with authentic samples where available, and by the preparation of suitable derivatives. Very small quantities of L-galactose and 2-O-methyl-L-galactose were isolated from oligosaccharides obtained after acetolysis of the polysaccharide (see later). In addition, paper-chromatographic evidence for the presence of traces of xylose, mannose and 6-O-methylgalactose was obtained; the latter was isolated in trace quantity from the partial hydrolysis of the polysaccharide, and optical rotation measurements indicate that at least the D-isomer is present.

The main component sugars in the polysaccharide were estimated quantitatively by GLC of their derived alditol acetates [122]. Since there is a variation in the relative molar responses of alditol acetates when using a flame ionization detector, this parameter had to be determined by means of standard solutions of each monosaccharide component, using the internal standard method. Erythritol was chosen as the internal standard since neither it nor erythrose was a constituent of the polysaccharide, and since the derived acetate has a suitable retention time on the gas chromatograph. The molar proportion of galactose: 4-O-methylgalactose : 2-O-methylgalactose, was 10.5 : 1.1 : 1.0. This gives the sulphated polysaccharide a theoretical methoxyl content of 2.46%, which is slightly higher than the 2.07% obtained experimentally using the Zeisel method [133].

The sulphate content of the polysaccharide (SO_4^{2-}, 19.9%) was determined by the 4'-chlorobiphenyl-4-ylamine method [116, 117]. This involves converting the sulphate hemi-ester groups in the polysaccharide into ionic sulphate

which is complexed with the above reagent. The absorbance of the above reagent is measured on a UV-spectrophotometer both before and after complexing with the sulphate, whence the amount of sulphate present can be readily determined from a standard graph.

Alkaline elimination of sulphate hemi-ester groups attached to both monosaccharides and polysaccharides can occur only under certain conditions. Thus when the sulphate hemi-ester group is attached to the 6-position of a hexose moiety with the 3-position free, or *vice versa*, elimination of sulphate can take place with concomitant formation of a 3,6-anhydride. Alternatively, if the sulphate hemi-ester group is adjacent and *trans* to a free hydroxyl group, then elimination (using sodium methoxide) can take place with the intermediate formation of an epoxide ring, followed by attack of methoxide ion on either side and *trans* to the epoxide oxygen, to form monomethyl sugars. Depending on steric factors, Walden inversion may or may not take place. This reaction can thus be used to help locate the positions of the sulphate ester groups, especially if potassium borohydride is added to minimize degradation of the polysaccharide chain [42]. When this method was applied to *Aeodes ulvoidea* polysaccharide, only *ca.* 10% of the sulphate was lost, resulting in the formation of an equivalently small amount of 3,6-anhydrogalactose. Thus although the polysaccharide contains a large amount of sulphate, it must be situated in such a way that suitable hydroxyl groups are blocked by glycosidic linkages, methyl ethers and/or sulphate ester (i.e. multisulphated units). In this respect the polysaccharide resembles the other

Grateloupiaceae polysaccharides thus far examined [93, 96, 98], although in the case of aeodan slightly more sulphate was eliminated [93].

Further information on the positions of the sulphate hemi-ester groups can be obtained by comparative periodate oxidation and methylation studies on the native and desulphated polysaccharides, and desulphated polysaccharide was prepared with this end in view. Thus the polysaccharide was desulphated by shaking with methanolic hydrogen chloride for 48 h, after which the solid was removed by filtration and retreated with fresh methanolic hydrogen chloride. This afforded a considerably degraded, desulphated polymer (SO_4^{2-}, 6.2%) in 69% yield calculated from sulphated starting material.

Paper chromatography of a neutralised, acid hydrolysate of the methanol-soluble material showed that desulphation had removed some galactose, 2-O-methylgalactose and 4-O-methylgalactose residues. Paper chromatography of a neutralised, acid hydrolysate of the desulphated polymer showed that the 4-O-methylgalactose spot was less intense than the 2-O-methylgalactose spot, whereas, for the unmodified polysaccharide the intensities of the spots were clearly reversed. This was confirmed by GLC studies of the derived alditol acetates. Although exact measurements were not possible, the estimated molar ratios of galactose : 4-O-methylgalactose : 2-O-methylgalactose were 35 : 1 : 2 (cf. corresponding molar ratios of 10.5 : 1.1 : 1.0 in the unmodified polysaccharide). This indicates that either the linkages of the monomethylgalactoses are more easily split than the galactose linkages or else (more likely) that the monomethylgalactoses,

and especially the 4-O-methylgalactose, occur at positions in the molecule which are more susceptible to bond cleavage under the reaction conditions e.g. at or near terminal units on the macromolecule (see later). Oxidations of polysaccharide and desulphated polysaccharide with sodium metaperiodate were followed spectrophotometrically [124] to completion (Table III). Although the desulphated polysaccharide consumed ca. 50% more periodate than the polysaccharide, this is readily explained by the extensive degradation which took place during desulphation of the polysaccharide. Paper chromatography of neutralised, acid hydrolysates of the oxopolysaccharides derived from both polysaccharide and desulphated polysaccharide revealed galactose and 2-O-methylgalactose, but no trace of 4-O-methylgalactose.

TABLE III : PERIODATE REDUCED (MMOLES) PER "ANHYDROHEXOSE" UNIT.

Time (h)	5	10	24	48	72	96	150
Polysaccharide[a]	100	-	228	246	264	264	-
Polysaccharide[b]	194	229	263	280	288	300	300
Desulphated polysaccharide (SO_4^{2-}, 6.2%)[a]	295	-	361	395	430	430	-

[a]Spectrophotometric determination.

[b]Titrimetric determination.

In order to obtain sufficient oxopolysaccharide for further examination, a second experiment was performed in which the oxidation with sodium metaperiodate was followed titrimetrically [125] (Table III). GLC studies of this oxopolysaccharide, using the same technique as mentioned earlier, showed no trace of 4-O-methylgalactose, the molar ratio of galactose : 2-O-methylgalactose in the oxopolysaccharide being 6.1 : 1. If all the 4-O-methyl-L-galactose residues in the polysaccharide are oxidised by periodate, they must be either (a) 1,6-linked or (b) linked only through either position one or six i.e. the 4-O-methyl-L-galactose must be present as either non-reducing or reducing end group. The possibility of linkage through position six only is ruled out by the molar ratio of 4-O-methyl-L-galactose residues to total anhydrohexose residues, i.e. ca. 1 : 10, since an absolute requirement of this would be an average DP of 10 for the polysaccharide. The possibility of 1,6-links is ruled out by the absence of 2,3,4-tri-O-methylgalactose in the hydrolysate of methylated, desulphated polysaccharide (see later). On the other hand, the presence of 4-O-methyl-L-galactose as non-reducing end-group could account, in part, for its ready removal during desulphation of the polymer. The mode of linkage of 4-O-methyl-L-galactose in two oligosaccharides, isolated from a partial hydrolysate of the polysaccharide, has been proven conclusively (see later).

The molar ratio of galactose to 2-O-methylgalactose in the oxopolysaccharide is also significant. The reduction of 0.3 mole of periodate per "anhydrohexose" unit by all the 4-O-methyl-L-galactose and part of the galactose in the

polysaccharide would leave the molar ratio of galactose : 2-O-methylgalactose at ca. 7 - 8 : 1, which is of the order of the ratio found. Hence, it appears that the 2-O-methylgalactose is largely immune to periodate attack. The majority of the 2-O-methylgalactose residues must therefore be either 1,3- and/or 1,4-linked. The possibility of 1,6-linked 2-O-methylgalactose, either without sulphate or sulphated in either position 3 or 4, is further ruled out by the absence of 2,3,4-tri-O-methylgalactose in the hydrolysate of the methylated, desulphated polysaccharide (see later). The mode of linkage of the 2-O-methylgalactose in four disaccharides and two trisaccharides has been proven conclusively (see later). Finally, the low reduction of periodate (0.3 mole of periodate) suggests that at least one half of the galactose residues are 1,3-linked and/or a large proportion of the galactose residues contain other glycosidic linkages so as to render them immune to periodate. The low percentage of alkali-labile sulphate in the polysaccharide precludes the possibility of a large proportion of 1,4-linked galactose residues protected from periodate attack by sulphate ester groups at positions 2 and/or 3.

The classical procedure for the determination of the glycosidic linkages in a polysaccharide, is methylation of all the free hydroxyl groups in the polysaccharide, followed by examination of the permethylated saccharides in the hydrolysate. However, difficulties arise when this procedure is applied to sulphated galactans. Thus when the permethylated saccharides, from a hydrolysate of a fully methylated sulphated polysaccharide, are examined, it is impossible to establish

which of the free hydroxyl groups were involved in either glycosidic or sulphate hemi-ester bonds. This difficulty can be partially overcome by comparing the permethylated saccharides obtained from hydrolysates of methylated fully sulphated and desulphated polysaccharides. However a great deal of caution is still necessary since (a) considerable glycosidic bond scission takes place during desulphation of the polysaccharide and (b) highly sulphated polysaccharides are notoriously difficult to fully methylate, due to steric and other factors. Since some difficulty was experienced in the methylation of even partially desulphated A. ulvoidea polysaccharide (SO_4^{2-}, 6.2%), no attempt was made to methylate the fully sulphated polysaccharide.

Methylation of the desulphated polysaccharide (SO_4^{2-}, 6.2%) was effected by a number of additions of solid sodium hydroxide and dimethyl sulphate to a solution of the polymer in dimethyl sulphoxide. Paper chromatography of a neutralised, acid hydrolysate showed mostly 2,3,6- and 2,4,6-tri-O-methylgalactoses, with smaller proportions of 2,3,4,6-tetra-O-methylgalactose, some di-O-methylgalactoses, and 2-O-methylgalactose. In view of the difficulty of methylating polysaccharides on a large scale with Purdie's reagents [126], only a small quantity of the methylated, desulphated polysaccharide (A) was exhaustively methylated in this way. The derived solid foam (B) showed no hydroxyl peak in the infrared, and had a methoxyl content of 37.4%. Paper chromatography of a neutralised, acid hydrolysate of B showed all the saccharides present in A, including the mono- and di-O-methylgalactoses in much the same concentration as for A. It was therefore decided

not to treat the bulk of A with Purdie's reagents, since it appeared to be already fully methylated.

Most methylated polysaccharides are insoluble in hot aqueous solution and therefore cannot be hydrolysed with dilute mineral acid. It is also important that the demethylation and degradation that take place during the hydrolysis should be kept at the lowest possible level. One way of achieving this is by partial formolysis, followed by hydrolysis of the resultant formate ester in aqueous mineral acid (134). This method was used for the above methylated polysaccharide, A. Separation of the hydrolysate by elution from a charcoal-Celite column with a linear gradient of 0 - 5% butanone in water, gave 2,3,6- and 2,4,6-tri-O-methyl-D-galactose as the major components, together with a smaller amount of a mixture of 2,3,4,6-tetra-O-methyl-D- and -L-galactose in the ratio of ca. 1 : 1 (inferred from an $[\alpha]_D$ value of + 2°). In addition, a mixture of methylated saccharides, identified by paper chromatography as 2-O-methylgalactose and a number of di-O-methylgalactoses, all in trace quantities, was obtained. The presence of 2,4,6-tri-O-methyl-D-galactose as a major saccharide in the hydrolysate supports the evidence cited earlier for 1,3-linked galactose residues, while the presence of 2,3,6-tri-O-methyl-D-galactose as the other major saccharide indicates a high proportion of 1,4-links in the macromolecule. The 2,3,4,6-tetra-O-methyl-D-galactose is considered to have arisen mainly from D-galactose and possibly from some of the 2-O-methyl-D-galactose end-groups. The presence of 4-O-methyl-L-galactose as non-reducing end-group in the desulphated polymer accounts for

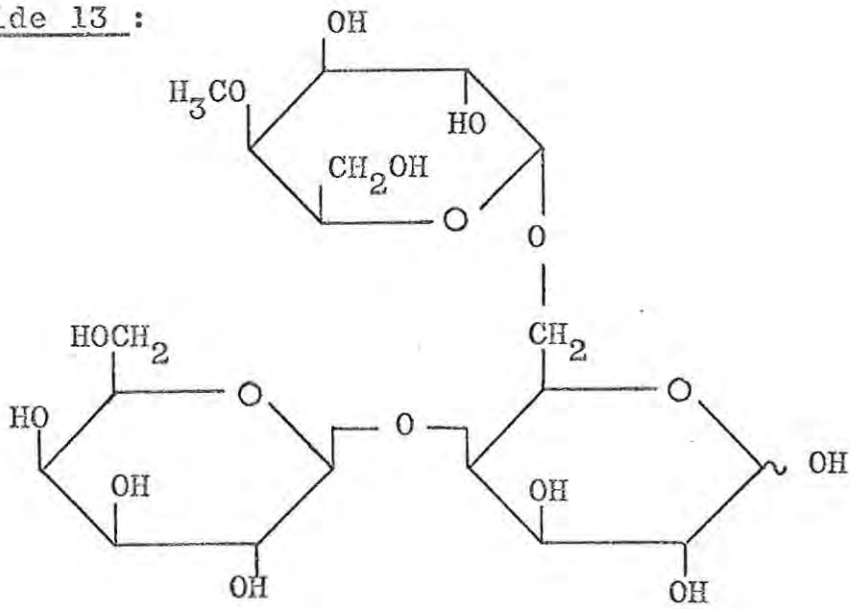
the presence of 2,3,4,6-tetra-O-methyl-L-galactose in the methylated, desulphated polymer. The contribution of L-galactose and 2-O-methyl-L-galactose in this connection is negligible since they are present only in trace quantity (see later). The presence of the 2-O-methylgalactose in the hydrolysate of the methylated polymer can be explained as follows. A. ulvoidea polysaccharide has been shown to contain pyruvic acid [135], and since its mode of linkage in algal polysaccharides [30, 102] has always been as the 4,6-acetal on anhydrohexose residues, VII, it is reasonable to assume that it is similarly linked in the present polysaccharide. Furthermore, since the acetal is sufficiently stable for it to be present linked to disaccharides isolated from acidic partial hydrolysates of polysaccharides [102], and since the conditions of desulphation of the present polymer were not very forcing, it is likely that at least some of the pyruvate would have survived desulphation conditions. If the hexose 4,6-acetal is glycosidically linked through the 3-position (as it is in agar [30]), then methylation of these units would give only 2-O-methylgalactose.

Partial hydrolysis of the polysaccharide with dilute mineral acid, followed by neutralisation and deionization, afforded a neutral syrup which was fractionated by elution from a charcoal-Celite column using aqueous ethanol. A total of 15 major fractions were collected, and where necessary these were fractionated by paper chromatography using a suitable solvent.

In addition to the monosaccharides already mentioned, five disaccharides and one trisaccharide were characterised.

Only two of the oligosaccharides obtained were crystalline, viz. 4-O-β-D-galactopyranosyl-D-galactose (2), and 4-O-β-D-galactopyranosyl-2-O-methyl-D-galactose (7). The other oligosaccharides were obtained as chromatographically pure syrups and have been identified as 6-O-β-D-galactopyranosyl-D-galactose (1), 6-O-α-(4-O-methyl-L-galactopyranosyl)-D-galactose (4), 4-O-β-(6-O-methyl-D-galactopyranosyl)-2-O-methyl-D-galactose (8) and O-α-(4-O-methyl-L-galactopyranosyl)-(1→6)-O-[β-D-galactopyranosyl-(1→4)]-D-galactose (13).

Oligosaccharide 13 :



The general methods used for the identification of these oligosaccharides were as follows. The component sugars in the oligosaccharide were determined by hydrolysis of the oligosaccharide followed by neutralisation, centrifugation, deionization and paper chromatography of the derived syrup. For those oligosaccharides which consisted of more than one sugar, reduction of the oligosaccharide (sodium borohydride) followed by hydrolysis and paper chromatography, using a spray reagent which revealed only reducing saccharides, clearly

indicated which saccharide was on the reducing end of the oligosaccharide. Oligosaccharides with DP > 2 were also subjected to partial hydrolysis, and reduction and partial hydrolysis, thus enabling the order of linkages in the oligosaccharide to be established. The position of the glycosidic linkage in the disaccharides was determined by methylation of the oligosaccharide using either a modified Hakomori procedure [130,131] or else the method of Haq and Percival [132]. In the former, a mixture of the oligosaccharide, dimethyl sulphoxide and sodium hydride were ultrasonicated for 30 min under nitrogen, followed by addition of methyl iodide and ultrasonication to a clear solution. In all cases, one treatment was sufficient to ensure a fully methylated product while degradation was kept to a minimum. The permethylated oligosaccharides were hydrolysed and the hydrolysate examined by paper chromatography and TLC. The remaining hydrolysate was methanolysed and the derived methyl glycosides examined by GLC.

Oligosaccharides having 1,3- or 1,6-linkages on the reducing end are difficult to methylate since considerable alkali degradation takes place under the highly alkaline conditions of methylation. Rees [81] has shown that oligosaccharides containing galactose with a 1,3-linkage on the reducing end, are completely degraded when dissolved in saturated calcium hydroxide solution and left in an atmosphere of nitrogen, while 4-O-β-D-galactopyranosyl-D-galactose survived this treatment, at least in large part. This indicates that it is the alkaline conditions which cause the degradation, rather than the methylating conditions as such.

One way of overcoming this difficulty would be to reduce the oligosaccharide with sodium borohydride prior to methylation, the derived alditol being stable to the methylation conditions [128]. However in the case of galactose, it then becomes impossible to distinguish between 1,3- and 1,4-linkages, unless of course reduction is carried out with sodium borodeuteride and the methylation products examined by mass spectrometry [128]. The degree of alkali degradation of 1,3-linkages varies from one methylation procedure to another [99], and the modified Hakomori procedure [130,131] was adopted for the present work both for the low degree of alkali degradation and for its simplicity. All oligosaccharides which have been thus far examined in this laboratory and which contain 1,3- or 1,6-linkages on the reducing end, produce only one main degradation product which manifests itself as a peak on GLC of a methanolysate of the hydrolysed, methylated oligosaccharide, and with a relative retention time of T 3.72. This is all the more unfortunate since T 3.72 occurs midway between the peaks of methyl 2,4,6-tri-O-methylgalactosides (T 3.55 and 3.97) so that if degradation has been considerable then these peaks are all but obliterated.

A further disadvantage in the use of methyl glycosides is that any one methylated saccharide may produce several peaks on the GLC, corresponding to the various anomeric and ring forms of the saccharide. Interpretation of a chromatogram (especially in the quantitative sense) becomes rather difficult when two or more methylated saccharides have similar retention times, with consequent overlapping

and masking of their many peaks. Conversely, this same multiplicity of peaks makes the identification of any one saccharide that much more positive. For example, methyl 2,3,6-tri-O-methylgalactose produces four GLC peaks, and in the identification of this saccharide the relative retention times of all four can be established. Some workers [99] have suggested that the ratio of these peaks is dependent on the method of methylation and time of methanolysis, and to a certain extent is characteristic of the parent sugar. However the present work indicates that (within the compass of the sixteen oligosaccharides studied) this is not so, provided that the parent permethylated oligosaccharide is hydrolysed to its free component sugars prior to methanolysis. In this case the peak area ratios would be determined by the relative thermodynamic stabilities of the various anomeric and ring forms of the glycosides.

The anomeric configuration of the oligosaccharides follows from the specific rotations.

The isolation of oligosaccharide 4 is especially interesting since it establishes, for the first time, the mode of linkage of 4-O-methyl-L-galactose in a red algal polysaccharide. The structure of oligosaccharide 13 was elucidated as follows. Partial hydrolysis of the oligosaccharide gave, in additon to galactose and 4-O-methylgalactose, oligosaccharides 2 and 14. This immediately establishes that the structure must be either that suggested or else linear (i.e. α-4_L(1→6)-β-G_D(1→4)-G_D where 4_L = 4-O-methyl-L-galactose and G_D = D-galactose). Reduction of the oligosaccharide followed by partial hydrolysis (under a wide

range of conditions) gave galactose and 4-O-methylgalactose but no trace of either oligosaccharides 2 or 4. This indicates that the branched structure is the correct one. Methylation of the oligosaccharide followed by hydrolysis and methanolysis gave methyl 2,3,4,6-tetra-O-methylgalactoside as the only GLC peak with no trace of methyl 2,3-di-O-methylgalactoside, nor any tri-O-methylgalactosides. The most likely explanation of this is that the 1,6-linkage causes the reducing end of the branched oligosaccharide to degrade under the alkaline methylation conditions so that only 2,3,4,6-tetra-O-methylgalactose is formed from the two non-reducing end groups. The methylation results preclude the possibility of the linear structure for the oligosaccharide since, if it is linear, 2,3,4- and 2,3,6-tri-O-methylgalactosides should be present in the methanolysate of the hydrolysed methylated oligosaccharide, since a 1,4-linkage at the reducing end of an oligosaccharide survives the alkaline methylation conditions. The structure of oligosaccharide 13 is highly significant. Thus periodate oxidation studies on the native polysaccharide and methylation of the desulphated polysaccharide (see later), have determined that the 4-O-methyl-L-galactose occurs as non-reducing end-group in the polysaccharide. These facts, coupled with the structure for oligosaccharide 13, establish the fact that the 4-O-methyl-L-galactose exists as single unit side chains glycosidically linked to galactose at position 6 in the polysaccharide chain.

Oligosaccharide 1 establishes the presence of D-galactose residues linked through the 6 position.

The significance of the other oligosaccharides isolated

from the partial hydrolysis study will be discussed with those from the acetolysis of the polysaccharide.

It is known that aqueous hydrolysis and acetolysis have different selectivities for the various types of glycosidic linkage. Thus Rees [81] has shown that during partial hydrolysis the 1,3-linkages in λ-carrageenan are split much more rapidly than the 1,4-linkages so that 3-O-α-D-galactopyranosyl-D-galactose could not be readily detected as a product : acetolysis of the same polysaccharide, however, afforded the 1,3-linked disaccharide in almost five times greater yield than the 1,4-linked isomer. The 1,6-linkages [81] in dextran and yeast mannan are hydrolysed less rapidly than the co-occurring linkages but acetolysed more rapidly. It was thus decided to perform an acetolysis on A. ulvoidea polysaccharide in order to obtain further information about its fine structure.

Acetolysis of the native polysaccharide was effected by shaking it with a mixture of acetic anhydride, acetic acid and sulphuric acid for 100 h. The acetylated oligosaccharides were extracted with chloroform, evaporated to a syrup, and deacetylated with sodium methoxide. Charged material was removed by passing a solution of the oligosaccharides through a column of Amberlite IR 120 (H^+) resin, followed by Amberlite IRA 400 (CH_3COO^-). The neutral oligosaccharides were applied to a charcoal-Celite column and eluted with water, followed by aqueous ethanol (0 - 17%) using the gradient technique. Fractions were collected, sorted by paper chromatography, and recombined into 13 major fractions. Further fractionations were achieved by paper

chromatography. Thirteen oligosaccharides were obtained pure, including some of those already isolated by the partial hydrolysis study. These are listed in Table IV.

Oligosaccharide 10 was the only one obtained in the crystalline form. Significantly, disaccharides with 1,3-linkages were obtained in higher yield than those with 1,4-linkages, while no oligosaccharide with a 1,6-linkage was obtained. Of the four trisaccharides isolated only one had a 1,3-linkage on the reducing end. The only tetrasaccharide, containing only D-galactose, which was isolated (15) corroborates the findings of Rees [81] and confirms the selectivity of acetolysis. It is interesting to note that the only tetrasaccharide isolated from Pachymenia carnosa [136] (this polysaccharide has a basically similar structure to A. ulvoidea) was O-β-D-galactopyranosyl-(1→4)-O-α-D-galactopyranosyl-(1→3)-O-β-D-galactopyranosyl-(1→4)-D-galactose. When this latter oligosaccharide was subjected to paper chromatography along with oligosaccharides 14 and 15 (R_{gg} 0.19 and 0.29), it had a mobility intermediate between oligosaccharides 14 and 15 and corresponded to the trace oligosaccharide with R_{gg} 0.25 present in fraction XI. Since oligosaccharide 15 is about 30 times more abundant than the next most abundant tetrasaccharide from A. ulvoidea, and since oligosaccharide 15 is the only tetrasaccharide isolated by Rees [81] from acetolysis of λ-carrageenan, it is tempting to speculate on the reason(s) why acetolysis of P. carnosa yielded the tetrasaccharide it did, and not oligosaccharide 15. In this connection it should be noted that neither A. ulvoidea

TABLE IV : OLIGOSACCHARIDES FROM *A. ulvoidea*

Oligosaccharide	Total Hydrolysis products	Partial Hydrolysis Products	Partial Hydrolysis Products (after reduction)	Hydrolysis or methanolysis products of methylated oligosaccharide
(1) $G_D\beta(1\rightarrow 6)G_D$	G	G		A, D
(2) $G_D\beta(1\rightarrow 4)G_D$	G	G		A, C
(3) $G_D\alpha(1\rightarrow 3)G_D$	G	G		A, B
(4) $4_L\alpha(1\rightarrow 6)G_D$	G,4	G,4	4	A, D
(5) $2_D\alpha(1\rightarrow 3)G_D$	G,2	G,2	2	A, B
(6) $G_D\beta(1\rightarrow 4)2_L$	G,2	G,2	G	A, C
(7) $G_D\beta(1\rightarrow 4)2_D$	G,2	G,2	G	A, C
(8) $6_D\beta(1\rightarrow 4)2_D$	2,6	2,6	6	A, C
(9) $G_D\beta(1\rightarrow 4)G_D\alpha(1\rightarrow 3)G_D$	G	G,(2),(3)	G,(2)	A, B, C
(10) $G_D\alpha(1\rightarrow 3)G_D\beta(1\rightarrow 4)G_D$	G	G,(2),(3)	G,(3)	A, B, C
(11) $G_D\alpha(1\rightarrow 3)G_D\beta(1\rightarrow 4)2_D$	G,2	G,2,(3),(7)	G,(3)	A, B, C
(12) $2_D\alpha(1\rightarrow 3)G_D\beta(1\rightarrow 4)2_D$	G,2	G,2,(5),(7)	G,2,(5)	A, B, C
(13) $4_L\alpha(1\rightarrow 6)$ $G_D\beta(1\rightarrow 4)$ — G_D	G,4	G,4,(2),(4)	G,4	See discussion p. 105
(14) G-G-G-G	G(D,L 1:1)	discussion p.109		
(15) $G_D\alpha(1\rightarrow 3)G_D\beta(1\rightarrow 4)G_D\alpha(1\rightarrow 3)G_D$	G	G,(2),(3),(9),(10)	G,(2),(3),(10)	

Key : G = Galactose
2 = 2-O-methylgalactose
4 = 4-O-methylgalactose
6 = 6-O-methylgalactose

A = 2,3,4,6-tetra-O-methylgalactose
B = 2,4,6-tri-O-methylgalactose
C = 2,3,6-tri-O-methylgalactose
D = 2,3,4-tri-O-methylgalactose

polysaccharide nor λ-carrageenan were desulphated prior to acetolysis, while the polysaccharide from P. carnosa was partially desulphated (SO_4^{2-}, 11.5%).

The presence of oligosaccharide 14 establishes the hitherto unexpected existence of L-galactose in the polysaccharide, since a hydrolysate of the oligosaccharide showed that the galactose was present as a racemate. Partial hydrolysis of the oligosaccharide indicates that it probably has an alternating sequence of D- and L-galactose units. Whether this is true or not, it is interesting that no tetrasaccharide containing a D- and L-galactose ratio of 3 : 1 was found, since this is the tetrasaccharide which would have been expected had the L-galactose been randomly distributed throughout the polysaccharide chain. This suggests that the L-galactose is either concentrated in some portion of the polysaccharide chain, or that it exists in a contaminating polysaccharide with an alternating sequence of D- and L-galactose residues.

Oligosaccharide 6 establishes the presence of 2-O-methyl-L-galactose in the polysaccharide, and shows that it is linked in the same way as the D-isomer. Oligosaccharide 6 was also isolated from P. carnosa but insufficient material was available for full characterization. Oligosaccharide 12, which contains a D-galactose to 2-O-methyl-D-galactose ratio of 1 : 2, was obtained in much higher yield than any other trisaccharide containing 2-O-methyl-D-galactose. This indicates that there are regions of the polysaccharide which are comparatively rich in 2-O-methyl-D-galactose. An alternative explanation is that

the presence of the 2-O-methyl-D-galactose stabilizes the glycosidic linkages to acetolysis, thus greatly increasing the yield of this oligosaccharide. The existence of a separate polysaccharide containing D-galactose and 2-O-methyl-D-galactose in strictly alternating sequence is ruled out by the presence of oligosaccharide 11 in the acetolysate.

The presence of oligosaccharide 8 in the partial hydrolysate of the polysaccharide raises a number of interesting points. The amount of free 6-O-methylgalactose which could be isolated from this partial hydrolysate was only about one fifth of the amount of oligosaccharide 8 obtained. Furthermore, if the 6-O-methylgalactose were distributed randomly in the polysaccharide chain then the chances of it being linked to galactose rather than 2-O-methylgalactose are ca. 10 : 1 (i.e. the ratio of galactose : 2-O-methylgalactose in the polysaccharide). No oligosaccharide containing 6-O-methylgalactose and galactose was found. All these facts suggests that the majority of the 6-O-methylgalactose residues are β-1,4-linked to 2-O-methylgalactose residues in the polysaccharide.

A number of broad features emerge from the rest of the oligosaccharides isolated from the partial hydrolysis and the acetolysis. Periodate oxidation studies and methylation of the desulphated polysaccharide have established (see earlier) that there are approximately an equal number of 1,3- and 1,4-linkages in the polysaccharide. All the trisaccharides contain both of these linkages (except oligosaccharide 13) and the only tetrasaccharide containing only D-galactose had

these linkages in alternating sequence, which supports the case for an alternating sequence of 1,3 and 1,4-linkages in the polysaccharide. The presence of oligosaccharide 1 does not necessarily interrupt this sequence, since it is possible that there are single galactose residues linked 1,6 as branch points on the polysaccharide chain (cf. linkage of the 4-O-methyl-L-galactose). This is supported by the absence of 2,3,4-tri-O-methylgalactose in the hydrolysate of methylated desulphated polysaccharide.

In all those oligosaccharides in which 2-O-methyl-D- and -L-galactose occur, this saccharide is linked through position 4, and on the reducing-end it is always α-1,3-linked.

The gross features of the polysaccharide from A. ulvoidea are very similar to those of the other Grateloupiaceae polysaccharides which have been investigated (refs. 93, 94, 96, 97, 98, 100, 136 and 137). Thus the macromolecules consist of a backbone of mainly D-galactose in which most of the residues are α-1,3- and β-1,4-linked.

Table V lists the galactoses and monomethylgalactoses found in the Grateloupiaceae polysaccharides. The presence of L-galactose and 2-O-methyl-L-galactose in A. ulvoidea, of L-galactose in P. cornea, and of 2-O-methyl-L-galactose in P. carnosa, was not suspected until these trace saccharides were detected in oligosaccharides obtained from solvolysis of the respective polysaccharides. It is very difficult to establish the presence of a trace saccharide in a polysaccharide when its enantiomer is present to a far greater extent. Thus it seems highly likely, that if sufficiently sophisticated methods could be employed, then both the D- and

L-isomers of galactose and the three monomethylgalactoses would be found in all five of the Grateloupiaceae polysaccharides. All five polysaccharides are highly sulphated, and the sulphate is largely alkali stable.

TABLE V : SACCHARIDES FOUND IN THE GRATELOUPIACEAE POLYSACCHARIDES

Aeodes ulvoidea	Aeodes orbitosa	Phyllymenia cornea	Pachymenia carnosa	Grateloupia elliptica
G_D, G_L	G_D, G_L	G_D, G_L	G_D	G_D, G_L
$2_D, 2_L$	2_D	2_D	$2_D, 2_L$	2_L
4_L	4_L	4_L	$4_D, 4_L$	4_D
6_D	6_D	6_D	6_D	

Key : G = galactose, 2 = 2-O-methylgalactose, 4 = 4-O-methylgalactose and 6 = 6-O-methylgalactose.

Only in G. elliptica have significant amounts of 3,6-anhydro-D-galactose, and traces of 3,6-anhydro-L-galactose been found.

The only positive evidence for branching is the 1,6-linked 4-O-methyl-L-galactose in A. ulvoidea.

As an indication of the fine structures of the polysaccharides the following oligosaccharides have been isolated : (i) from A. orbitosa, oligosaccharide 2 and 3-O-D-galactopyranosyl-D-galactose, (ii) from P. cornea, oligosaccharides 2,7,8 and 4-O-β-D-galactopyranosyl-L-galactose

(iii) from *P. carnosa*, oligosaccharides 2,3,5,6,7,8,9,10,11, 12 plus seven other oligosaccharides.

No unique structure can be proposed for *A. ulvoidea* polysaccharide, since there is much masking of the basic D-galactose backbone, and because the position of the ester sulphate has not yet been determined.

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