"A STRUCTURAL INVESTIGATION OF THE SULPHATED POLYSACCHARIDES OF *Aeodes orbitosa* AND *Phyllymenia cornea*"

BY

HARALAMBOS PAROLIS B.Sc.(Pharm), B.Sc.(Hons)(Rhodes)

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SUMMARY

A highly sulphated, methylated polysaccharide, aeodan, isolated from the red seaweed Aeodes orbitosa was shown to contain galactose, 2-O-methyl-D-galactose, 4-O-methyl-L-galactose, 6-O-methyl-D-galactose, xylose, and glycerol. The polysaccharide was desulphated with methanolic hydrogen chloride. Periodate oxidation of aeodan and desulphated aeodan, followed by reduction and hydrolysis, revealed the presence of 1,4- and 1,3-linked galactose residues and 1,3-linked 6-O-methyl-D-galactose residues in aeodan. Treatment of aeodan with sodium hydroxide revealed that the majority of the ester sulphate groups were alkali stable. Methylation of desulphated aeodan revealed that the polysaccharide was composed entirely of 1,3 and 1,4 links. Methylation of aeodan revealed the presence of 1,3- and 1,4-linked units, 1,3-linked galactose-2-sulphate, and 1,3-linked galactose-2, 6-disulphate units in the polysaccharide. Partial hydrolysis of aeodan resulted in the isolation and characterisation of 3-O-D-galactopyranosyl-D-galactose and 4-O-β-D-galactopyranosyl-D-galactose.

A sulphated, methylated polysaccharide, phyllymenan, isolated from the red seaweed Phyllymenia cornea was shown to contain galactose, 2-O-methyl-D-galactose, 4-O-methyl-L-galactose, 6-O-methyl-D-galactose, and xylose. The polysaccharide was completely desulphated with methanolic hydrogen chloride. Periodate oxidation of phyllymenan before and after desulphation revealed that removal of the sulphate ester groups had not produced any new adjacent hydroxyl groups. Alkali treatment of phyllymenan revealed that the ester sulphate groups were alkali stable. Methylation studies on phyllymenan revealed the presence of 1,3- and 1,4-linked

units/.....

units, 1,3-linked galactose-2-sulphate, and 1,3-linked galactose-2,6-disulphate units in the polysaccharide. Partial hydrolysis of phyllymenan revealed the presence of 4-O-β-D-galactopyranosyl-D-galactose, 4-O-β-D-galactopyranosyl-2-O-methyl-D-galactose, a galactosylgalactose composed of D- and L-galactose, and adjacent 6-O-methyl- and 2-O-methyl-D-galactose units in the polysaccharide.

1. INTRODUCTION.

ALGAL POLYSACCHARIDES CONTAINING SULPHATE ESTERS

Polysaccharides containing sulphate hemi-ester groups can be obtained in copious amounts by hot water extraction of the seaweeds belonging to the Chlorophyceae (green algae), Rhodophyceae (red algae), and Phaeophyceae (brown algae). These polysaccharides are present in the continuous matrix of the cell wall as well as in the amorphous gel-like materials in which the cells are embedded. Their functions in the seaweed are, as yet, incompletely understood, but two obvious functions are related to their ion-exchange properties, and their ability to form gels. The algae, living in a saline environment, must possess a mechanism for ion-exchange with their environment in order that essential cations may be selectively absorbed. The gelling and hydrophilic properties of these polysaccharides obviously help to avoid desiccation of the algal cells when the seaweed is exposed at low tide and confers upon the seaweed a structure which is pliant and flexible thus contributing to the physical security of the alga.

The sulphate content of these polysaccharides varies from less than one per cent as in agar, to greater than twenty per cent as in the λ component of carrageenan. The presence of the sulphate group seriously handicaps structural investigations. For example, complete methylation of all the free hydroxyl groups of a sulphated molecule can only be achieved if the sulphate content is fairly low. It is extremely difficult even under forcing conditions to achieve complete methylation of a highly sulphated polysaccharide, presumably due to steric hindrance. In such cases evidence for the modes of linkage between the monosaccharide units and the location of the sulphate ester groups must be sought from partial degradation studies of the macromolecule and

also from/.....

also from methylation studies on the desulphated polymer.

1.1. Polysaccharide sulphates of red algae.

Polysaccharides of the Rhodophyceae can conveniently be divided into two main groups; (i) Polysaccharides containing an alternating chain of 1,3 and 1,4 links.
(ii) Miscellaneous polysaccharides. Polysaccharides belonging to the first group can be subdivided into agarose type polysaccharides and κ-carrageenan type polysaccharides.

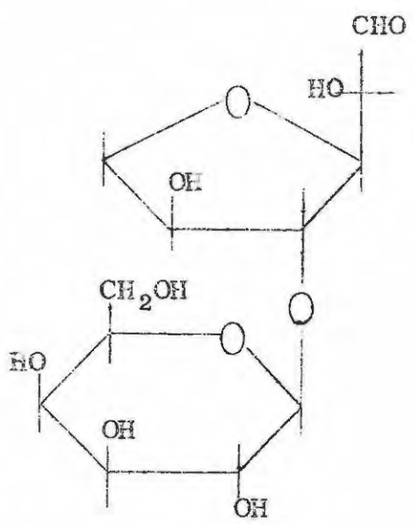
1.1.1. Agarose type polysaccharides.

1.1.1.1. Agar. Agar is extracted with hot water from several species of the Florideae. The predominant component is a galactan, with which is combined a small amount of sulphate. The predominant components of technically purified agar are D-galactose (50-65%), L-galactose (1%), 3,6-anhydro-L-galactose (30-50%), and ester sulphate (0.1-5%). In addition, the presence of small amounts of pentose and uronic acid have been reported. The constitution varies according to the source of the agar, that from Gelidium amansii having twice as much D-galactose as 3,6-anhydro-L-galactose, whereas that from Gracilaria confervoides contains these two sugars in equal amounts.

Percival and Somerville[1] isolated 2,4,6-tri-O-methyl-D-galactose, but no 2,3,4,6-tetra-O-methyl-D-galactose from the hydrolysate of methylated commercial agar. From a mixture obtained by remethylating hydrolysed methylated commercial agar a second sugar, methyl 2,4-di-O-methyl-3,6-anhydro-β-L-galactoside[2,3] was isolated. These studies established the presence of 1,3-linked D-galactose units and of 3,6-anhydro-L-galactose in agar. The glycosidic linkage to the anhydro-sugar in agar from Gelidium amansii was shown by Araki[4,5] to involve position-4. This follows from the isolation of

3,6-anhydro-2-O-methyl-L-galactose dimethyl acetal from a methanolysate of the methylated agar. Since anhydro ring formation is known to occur in the alkaline hydrolysis of some sugar sulphates[6-9], Jones and Peat[10] suggested that the anhydrosugar might be an artefact introduced during the methylation process. This, however, is not the case as agar does not contain sufficient ester sulphate to account for all the 3,6-anhydrosugar present. In addition, agar extracted under neutral or acid conditions still gives a product containing the anhydrosugar.

Further information on the structure of agar was obtained when Araki[5,11] isolated a crystalline disaccharide, agarobiose, from the agar of Gelidium amansii by partial acid hydrolysis. This he demonstrated to be 3,6-anhydro-4-O-(β-D-galactopyranosyl)-L-galactose (i).



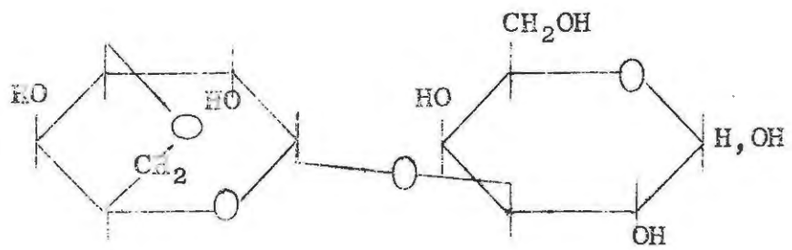
(i)

In addition, Araki[12] isolated agarobiose in the form of its dimethyl acetal in very high yield from a partial methanolysate of agar.

At a later stage Araki and Arai[13,14] isolated a crystalline disaccharide and an amorphous tetrasaccharide from agar which had been treated by an enzyme from the marine bacterium Pseudomonas kyotoensis. The disaccharide,

neoagarobiose,/.....

neoagarobiose, was shown by hydrolysis and methylation to be 3-O-(3,6-anhydro-α-L-galactopyranosyl)-D-galactose (ii).



(ii)

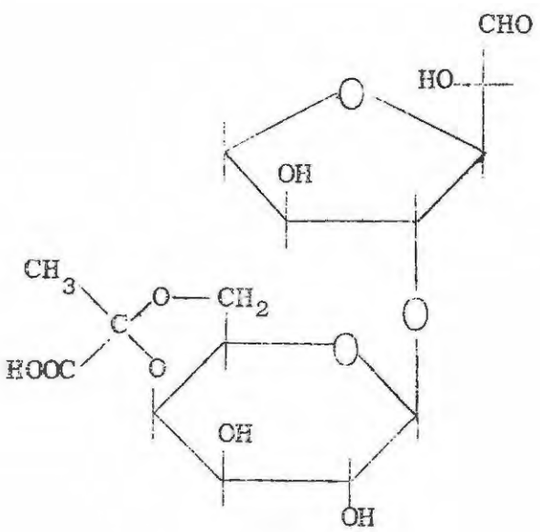
The tetrasaccharide, neoagarotetrose, was shown to consist of two neoagarobiose units linked β through the reducing group of one neoagarobiose unit to position-4 of the anhydrogalactose moiety of the second neoagarobiose unit. The isolation of agarbiose, neoagarobiose, and neoagarotetrose suggests that agar consists mainly of a linear polysaccharide containing alternating 3-linked D-galactose and 4-linked 3,6-anhydro-L-galactose units.[13,14] It has been claimed[15] that agar is composed of two polysaccharides, agarose, the acetate and methyl ether of which are soluble in chloroform, and agaropectin, having its acetate and methyl ether insoluble in chloroform. The structure postulated above refers to the principal polysaccharide, agarose. The agaropectin fraction is thought to be a complicated polysaccharide containing not only some of the features of agarose but also uronic acid, sulphate, and L-galactose. It is believed that the agarose chain is terminated at the non-reducing end by a 3,6-anhydro-L-galactose unit and at the reducing end by a D-galactose unit, since neither D-galactose nor 3,6-anhydro-L-galactose was detected in the enzymic hydrolysate of agar which involved cleavage of β-glycosidic links.

The proposed structure for agarose accords with the evidence for some agar specimens. Hydrolysis of methylated agarose would yield 60 per cent of 2,4,6-tri-O-methyl-D-

galactose/.....

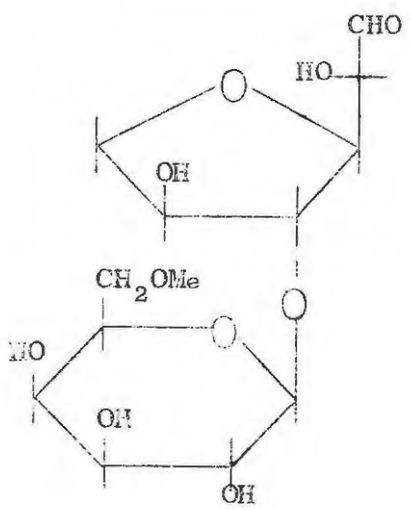
galactose. Percival and Thompson[16] obtained 65 per cent of 2,4,6-tri-O-methyl-D-galactose. Agarose would be resistant to periodate oxidation as has been observed for agar.[17]

Pyruvic acid has been reported as a minor constituent of certain specimens of commercial agar.[18] Methanolysis of one of these agar specimens gave, among other products, an acidic disaccharide containing pyruvic acid. The structure of the acidic disaccharide was established as (iii).



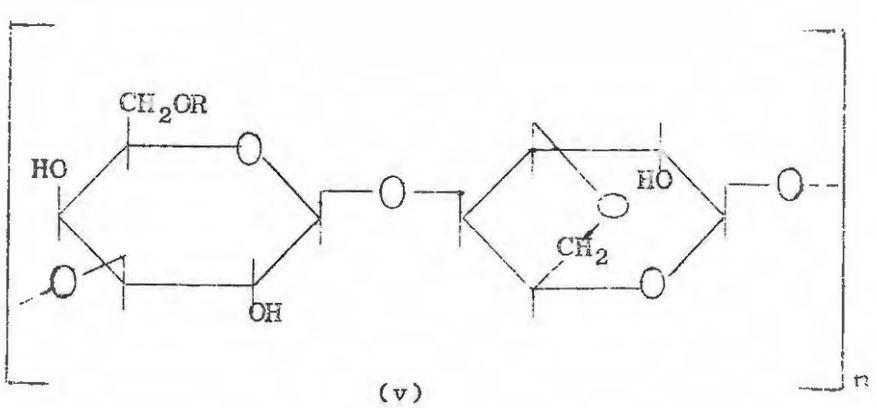
(iii)

Araki, in a recent study[19] on the structures of agars obtained from several species of agarophytes, has confirmed the presence of 6-O-methyl-D-galactose and 4-O-methyl-L-galactose as components of agaroses. The yield of 4-O-methyl-L-galactose from the hydrolysis products of the agar of G. amansii was so low that it was considered to have no structural significance. The 6-O-methyl-D-galactose content of the agaroses varies from one to twenty per cent. The mode of linkage of the 6-O-methyl-D-galactose units in agarose was solved when 3,6-anhydro-4-O-(6-O-methyl-β-D-galactopyranosyl)-L-galactose (iv) was isolated, in the form of its dimethyl acetal, from the partial methanolysate of the agar of Ceramium boydenii.



(iv)

This fact proves that the 6-O-methyl-D-galactose residues are glycosidically linked with position-4 of the 3,6-anhydro-L-galactose residues in exactly the same manner as the D-galactose residues. Although the proportions of D-galactose and 6-O-methyl-D-galactose vary widely in the different agaroses, Araki[19] noted that the sum of the amounts of these sugars always constitutes 51 to 53% of the polysaccharide. The 3,6-anhydro-L-galactose content is always 44 to 45% of any agarose. The molar ratio of the anhydrosugar to the sum of the above two sugars is very close to one in any agarose sample. It is possible, from all of the above-mentioned data, to set up the following repeating unit (v) for agaroses from various sources.



(v)

R varies/.....

R varies from unit to unit, but is usually H and less frequently CH_3.

1.1.1.2. Funori. The mucilage of the red alga Gloiopeltis furcata, funori, contains D-galactose, L-galactose, and 3,6-anhydro-L-galactose in the molar ratio 12:1:8 together with sulphate 18%.[20] The polysaccharide was shown to resemble agar when it gave a good yield of agarobiose dimethyl acetal on methanolysis, however it differs from agar in having a high sulphate content.

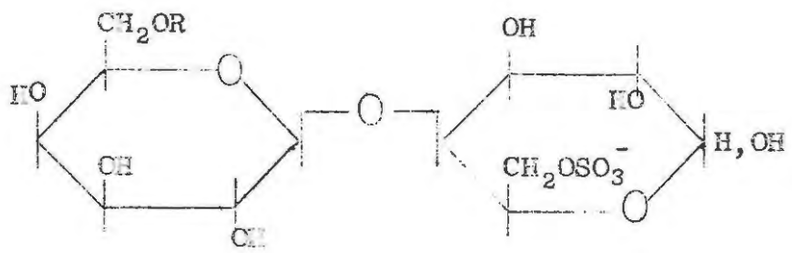
1.1.1.3. Porphyran. Porphyran is the name given to the water soluble mucilage of various species of Porphyra. The porphyran obtained by hot water extraction of P. umbilicalis[21] contained D- and L-galactose, 6-O-methyl-D-galactose, 3,6-anhydro-L-galactose, and ester sulphate in the molar ratio 100:57:35:65 while the porphyrans obtained from P. capensis[22] and P. linearis[23] contained these units in the molar ratio 100:100:200:100. The sulphate polysaccharide obtained from P. naiadum[24] differs from all the other porphyrans examined so far in that it does not appear to contain any 6-O-methylgalactose, but instead contains xylose.

Partial acid hydrolysis of porphyran led to the isolation of L-galactose-6-sulphate.[25] Alkali treatment of the porphyran of P. umbilicalis indicated that 86% of the sulphate is present as 1,2- or 1,4-linked L-galactose-6-sulphate.[26] In addition, infrared[26] and rate studies[25] on porphyran indicated the presence of a second sulphate. Treatment of porphyran with an enzyme extracted from the alga resulted in the removal of sulphate with concomitant production of 3,6-anhydro-L-galactose units. This suggests that L-galactose-6-sulphate is probably the biological precursor of 3,6-anhydro-L-galactose in porphyran.[27] Although the proportions of the components vary widely from sample to sample, Rees and Conway[28] noted that the sum of

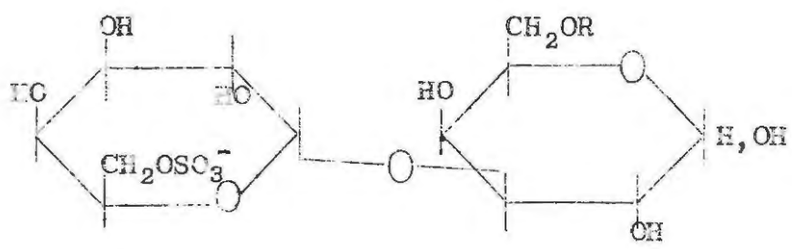
the proportions/...

the proportions of L-galactose-6-sulphate and 3,6-anhydro-L-galactose always equals the sum of the proportions of D-galactose and 6-O-methyl-D-galactose.

Partial acid hydrolysis of porphyran resulted in the isolation of four disaccharide monosulphates.[29] The structures are shown in (vi) to (ix).



(vi) R = H
(vii) R = CH_3



(viii) R = H
(ix) R = CH_3

A repeating sequence has been suggested for porphyran, in which 3-linked D-galactose units alternate with 4-linked L-galactose-6-sulphate residues, with the variations that some of the galactose carries an O-methyl group at position-6 and that some of the sulphated units have been desulphated to form 3,6-anhydro-L-galactose.[29] Further evidence for an alternating structure was obtained when derivatives of 2,4,6-tri-O-methylgalactose, 3,6-anhydro-2-O-methylgalactose, and tetramethylagarobiose were obtained from methanolysates of methylated alkali modified porphyran.[30] In a more recent study Christison and Turvey[31] degraded

porphyran/.....

porphyran with enzymes from a Cytophaga species. Neoagarabiose, neoagarotetrose, and significant amounts of a tetrasaccharide with the probable composition 3,6-anhydro-O-α-L-galacto-pyranosyl-(1-3)-6-O-methyl-O-β-D-galactopyranosyl-(1-4)-3,6-anhydro-O-α-L-galactopyranosyl-(1-3)-D-galactose were isolated. These studies[29,30,31] have confirmed the proposed sequence[29] for porphyran, and have shown the polysaccharide to be an elaborate version of agarose.

1.1.2. κ-Carrageenan type polysaccharides.

1.1.2.1. Carrageenan. The water soluble mucilages of many members of the Gigartinaceae contain a high proportion of a galactan sulphate, carrageenan. The principle commercial sources of carrageenan are Chondrus and Gigartina species. The polysaccharide contains 29-35% sulphate and 33-44% galactose, the constitution varying according to the species from which it is extracted. The galactose is present predominantly as the D-isomer.

It was long suspected that carrageenan was heterogeneous.[32,33] The cold and hot water extracts were found to have slightly different physical properties, the hot water extract setting to a gel but not the cold water extract. It was suggested that these differences might be accounted for by differences in the nature of the cations associated with the sulphate.[33]

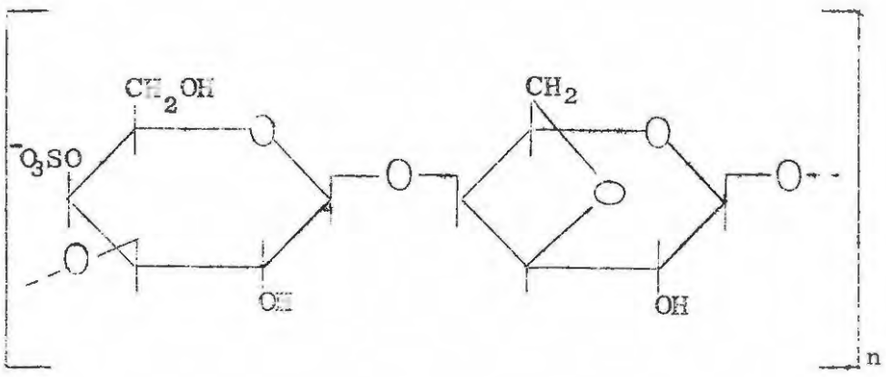
Hydrolysis of methylated carrageenan from Chondrus crispus with dilute oxalic acid[33,34] afforded 2-O- and 2,6-di-O-methyl-D-galactose. Since the sulphate groups in the original carrageenan were essentially alkali stable, it was concluded that sulphate was attached to position-4, and hence position-3 was glycosidically linked. In addition, the 2-O-methyl-D-galactose isolated was considered to have arisen from branching in the macromolecule, position-6 being the point of attachment of these branches.[35] Confirmation of

this structure/.....

this structure was obtained when a partially degraded desulphated carrageenan was methylated to give 2,4,6-tri-O-methyl-D-galactose and some 2,6-di-O-methyl-D-galactose on hydrolysis.[35,36]

In 1953 carrageenan was shown to be a mixture of at least five polysaccharides.[37,38,39] It was separated into two main polysaccharides, κ-carrageenan (40%), which gelled at a concentration of 0.15M potassium chloride and λ-carrageenan, (45%) which was soluble in this reagent.[37,40]

O'Neill,[41,42] using the technique of mercaptolysis, isolated the diethyl mercaptals of D-galactose and 3,6-anhydro-D-galactose from κ-carrageenan. In addition, a disaccharide mercaptal was obtained from a partial mercaptolysate. The free disaccharide, carrabiose, was shown to be 3,6-anhydro-4-O-(β-D-galactopyranosyl)-D-galactose. The ratio of D-galactose to 3,6-anhydro-D-galactose in κ-carrageenan is approximately unity, and it has been suggested that this component consists of alternating units of D-galactose-4-sulphate linked to 3,6-anhydro-D-galactose as shown below in (x).[42]



(x)

Enzymic hydrolysis of κ-carrageenan by a κ-carrageenase[43] isolated from Pseudomonas carrageenovora produced a homologous series of sulphated oligosaccharides with 3-O-(3,6-anhydro-α-D-galactopyranosyl)-D-galactose-4-sulphate as the major degradation product, and an enzyme resistant fraction. The

enzyme/.....

enzyme resistant fraction after treatment with alkali was degraded by κ-carrageenase. This study, therefore confirms the alternating structure (x) as the major structural unit, but also provides evidence for the presence of alkali-labile sulphate units in κ-carrageenen.

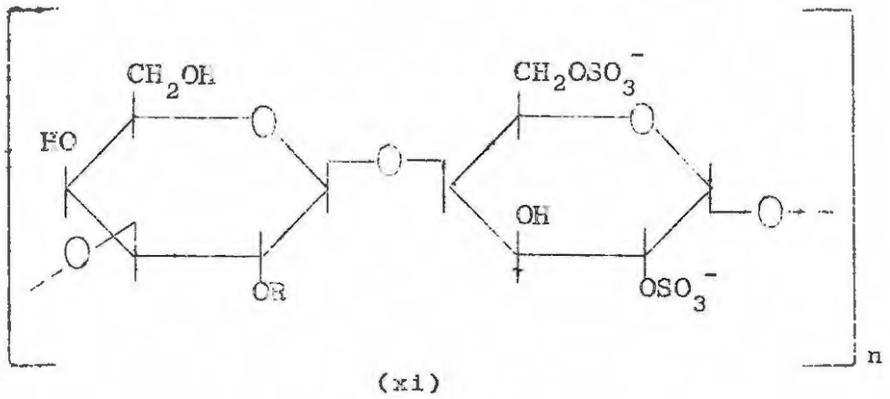
Methylation studies[44] on κ-carrageenan have shown that all the 3-linked galactose residues are 4-sulphated, and that a proportion of the 3,6-anhydrogalactose units are 2-sulphated. Other studies[45] have shown that a small proportion of galactose units are 4-linked. Some of these units carry sulphate at position-6 while others carry sulphate at positions-2 and -6. The presence of all these various sulphates has been confirmed by partial hydrolysis studies[46] on κ-carrageenan prepared from Chondrus crispus.

The structure of λ-carrageenan is more complex than the κ-component, and has until recently been very poorly understood. The presence of 1,3-linkages was demonstrated when 3-O-α-D-galactopyranosyl-D-galactose was isolated from an acetolysate of λ-carrageenan.[47] Rees has shown that the λ-carrageenan from Chondrus crispus contains one-third of its sulphate ester groups so placed on the galactose units that treatment with alkali, in the presence of borohydride,[26] removed them with concomitant formation of 3,6-anhydrogalactose units.[48] The latter units were identified by the isolation of the crystalline diethyl dithioacetal derivative after hydrolysis of the polysaccharide. Partial mercaptolysis of the alkali-treated λ-carrageenan gave carrabiose diethyl dithioacetal, thus displaying the presence of 1,4-linkages in λ-carrageenan. Mild acid hydrolysis of alkali-modified λ-carrageenan, followed by reduction with borohydride, yielded a degraded polysaccharide, which when subjected to a Smith degradation gave a sugar sulphate tentatively identified as 3,6-anhydro-D-galactitol-2-sulphate.[48] The above work indicates the

presence of/.....

presence of 4-linked D-galactose-2,6-disulphate units in λ-carrageenan. A study[48] of the rates of sulphate ester removal from alkali-modified λ-carrageenan by acid hydrolysis tends to confirm the presence of galactose-4-sulphate and 3,6-anhydrogalactose-2-sulphate units.

In a recent study Rees[49] has deduced the positions of the glycosidic linkages by the isolation of 2,3,4,6-tetra-, 2,4,6-tri-, and 2,3,6-tri-O-methyl-D-galactose from a hydrolysate of desulphated λ-carrageenan. Evidence for the location of the sulphate groups in λ-carrageenan was obtained when 2,4,6-tri-, 4,6-di-, 2,6-di-, and 3-O-methyl-D-galactose were isolated from methylated sulphated λ-carrageenan. The above results provide evidence for the presence of 1,4-linked galactose -2, 6-disulphate and 1,3- linked galactose -2-, and -4-sulphates in λ-carrageenan. Rees[49] has defined λ-carrageenan as a molecule (xi) devoid of 4-sulphate and 3,6-anhydrogalactose.



(xi)

R varies from unit to unit, but is usually SO_3^-, and less frequently H. Support for the above definition came when alkali-modified λ-carrageenan[49] was separated into two fractions, one of which was shown by methylation analysis to contain all the galactose-4-sulphate units. Rees believes that the λ-carrageenan examined by him is the biological precursor of κ-carrageenan.

1.1.2.2. Iridophycin./.....

1.1.2.2. Iridophycin. Iridophycin is a galactan sulphate obtained from red algae of Iridophycus spp.[50] The presence of 1,3 - linkages in the polysaccharide was proved by Mori[51] when he isolated 2,4,6-tri-O-methyl-D-galactose from the hydrolysate of a methylated desulphated iridophycin. Mori[52] is of the opinion that the sulphate group is attached to position-6, since the polysaccharide is resistant to tritylation. Iridophycin probably is a mixture containing some κ-carrageenan, since extracts of an Iridophycus species were hydrolysed by an enzyme specific for κ-carrageenan.[53] In a later study Yaphe[54] detected 36% of a κ-carrageenan type polysaccharide in iridophycin.

1.1.2.3. Hypnea spicifera polysaccharide. Hot water extraction of this red alga[55], followed by addition of potassium chloride, afforded a κ-carrageenan type polysaccharide, which contained galactose, 3,6-anhydrogalactose, and sulphate ($NaSO_3^-$) in the molar ratio 1.4:1.1:1.0. Partial hydrolysis followed by reduction afforded 3,6-anhydro-4-O-β-D-galactopyranosyl-D-galactitol, thus establishing the presence of 1,4-linkages in the macromolecule. Methylation of the polysaccharide indicated the presence of 1,3-linked galactose-4-sulphate.

1.1.2.4. Furcellaran. Extraction of Furcellaria fastigiata yields a galactan sulphate, furcellaran (Danish Agar), containing D-galactose, 3,6-anhydro-D-galactose, and ester sulphate (19%). Methylation of a desulphated degraded polysaccharide, followed by hydrolysis, yielded 2,3,4,6-tetra-, 2,4,6-tri-, and 2,4-di-O-methyl-D-galactose.[56] This suggests the presence of 1,3-linked galactose units and branching through position-6. Mercaptolysis of furcellaran yielded the mercaptals of D-galactose, 3,6-anhydro-D-galactose, and carrabiose.[57]

Partial hydrolysis/.....

Partial hydrolysis studies[46] on commercial furcellaran led to the isolation of D-galactose-2-sulphate, D-galactose-4-sulphate, D-galactose-6-sulphate, and 3,6-anhydro-D-galactose-2-sulphate. The same compounds were detected in the autohydrolysate of furcellaran prepared from Furcellaria fastigiata. These results suggest that the sulphate ester groups in furcellaran are more or less randomly distributed over all the available hydroxyl groups. The above-mentioned data, coupled with the ability of the polysaccharide to gel in the presence of potassium chloride, indicates that furcellaran possesses a structure similar to that of κ-carrageenan.

1.1.2.5. Mucilage of Eucheuma spp. The barium salt of the cold water extract of Eucheuma muricatum[58,59,60] contains xylose, anhydrogalactose, and galactose in the molar ratio 1:3.5:12.5 together with sulphate and glucuronic acid (3.27%). At present there is no structural evidence available.

1.1.3. Miscellaneous polysaccharides.

1.1.3.1. Polysiphonia fastigiata. Dilute acid extraction of this alga gives a mucilage which contains galactose (D:L ratio 2:1), 6-O-methylgalactose (D:L ratio 9:7), 3,6-anhydrogalactose (D:L ratio 1:1), ester sulphate, and xylose.[61] Partial hydrolysis of the polysaccharide afforded a sugar sulphate which was tentatively identified as a mixture of D- and L-galactose-6-sulphate. Complete methylation of the polysaccharide, followed by hydrolysis, gave 2,4-di-O-methylgalactose (both D and L forms) as the main product. The dimethylgalactose probably arose from 3-linked galactose-6-sulphate units. At present no other structural information is available.

1.1.3.2. Dilsea edulis. Extraction of this alga with water or dilute acid yields a polysaccharide which contains

D-galactose (70%), D-xylose (7%), D-glucuronic acid (10%), sulphate (9.7%), and a trace of 3,6-anhydrogalactose.[62,63,64] Methylation of the desulphated polysaccharide, followed by methanolysis, gave the methyl glycosides of 2,3,4,6-tetra- and 2,4,6-tri-O-methyl-D-galactose together with traces of 2,3,6-tri- and a di-O-methylgalactose,[62] thus indicating the presence of predominantly 1,3-linkages in the macromolecule. Application of the Barry degradation technique[65] indicated that the polysaccharide contains a periodate resistant core of 1,3-linked galactose sulphate units. Examination of the low-molecular weight products from the degradation procedure led to the postulation of a repeating unit of thirteen 1,3-linked galactose units, four of which carry sulphate at position-6, to which four side chains containing both 1,3- and 1,4-linked galactose units, 1,3-linked xylose units, with 3,6-anhydro-galactose and glucuronic acid as end groups,[63,64] are attached.

Rees[66] has recently re-examined the mucilage of Dilsea edulis, and has shown that most of the sulphate is attached to position-4 of the galactose units. In addition, alkali treatment of the polysaccharide before and after periodate oxidation indicates the presence of some 1,4-linked galactose-6-sulphate units. Rees considers the xylose to be present as part of a contaminating polysaccharide. The polysaccharide consists of two structurally dissimilar regions. The major component is a chain of 1,3-linked galactopyranosyl units (with the possibility of some branching) with sulphate occurring on position-4 of some of these. The second region is an alternating chain of 1,3- and 1,4-linked galactose units, some of the 1,3-linked units perhaps carrying sulphate at position-4, and some of those linked 1,4 occurring as the 6-sulphate or 3,6 anhydride.

1.1.3.3. Mucilage of Dumontia incrassata. Dilute acid extraction of this red alga gives a water soluble polysaccharide containing galactose, sulphate, and uronic acid in the molar ratio [67] of 9:4:1. Periodate oxidation indicates that most of the galactose is linked 1,3, but, in addition, there may be present a few 1,4-linkages.

1.1.3.4. Corallina officinalis. Hot water extraction of this calcareous red alga under faintly acid conditions, followed by precipitation with cetylpyridinium chloride, yielded a polysaccharide containing galactose (D:L ratio, 1·3:1), D-xylose, and sulphate (SO_3Na) in the molar ratio of 4.2:1.9:1.[68] No 3,6-anhydrogalactose was detected in the polysaccharide. Partial hydrolysis of the polysaccharide led to the isolation and characterisation of L-galactose-6-sulphate and galactose-4-sulphate. Periodate oxidation of the polysaccharide resulted in the cleavage of all of the xylose residues, most of the L-galactose residues, and about two-thirds of the D-galactose residues. These results indicate that only a few 1,3-linked galactose residues are possible in the structure. At present no other structural information is available.

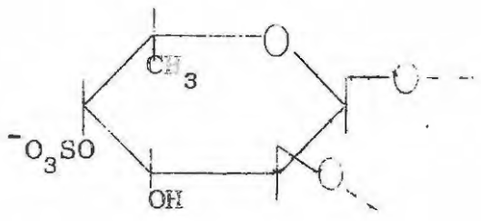
1.2. Sulphated polysaccharides of brown algae.

1.2.1. Fucoidin. The principal polysaccharide sulphate ester of the Phaeophyceae, fucoidin, yields , on acid hydrolysis, L-fucose as the major monosaccharide unit. Fucoidin was first described and named by Kylin,[69] who isolated the substance from various species of Laminaria and Fucus by extraction with dilute acetic acid. In addition to L-fucose, some specimens of fucoidin contain small amounts of galactose and xylose. Fractionation of a specimen of fucoidin from Ascophyllum nodosum on diethylaminoethyl

cellulose gave/.....

cellulose gave a xylan together with several fractions, which contained both fucose and galactose units. These fractions differed from each other in sulphate content.[70] These results suggest that galactose, but not xylose, is an integral part of the fucoidin molecule. Schweiger[71] has obtained similar evidence from the fucoidin of Macrocystis pyrifera, which it is suggested contains residues of L-fucose and D-galactose in the molar ratio 18:1.

The complete structure of fucoidin is not yet established. Conchie and Percival[72] elucidated certain of the structural features of the fucoidin from Fucus vesiculosus. Methylation followed by complete acid hydrolysis produced L-fucose (one part), 3-O-methyl-L-fucose (three parts), and 2,3-di-O-methyl-L-fucose (one part). Since most of the sulphate groups present in fucoidin are stable to alkali, the isolation of 3-O-methyl-L-fucose in high yield suggests a predominance of fucose-4-sulphate units linked through positions-1 and -2 (xii). The alternative unit, fucose-2-sulphate linked through positions-1 and -4, possesses a hydroxyl group adjacent and trans to a sulphate group and is thus available for anhydro ring formation with concomitant elimination of sulphate when treated with alkali.[73]



(xii)

The presence of 2,3-di-O-methyl-L-fucose indicates the presence of 1,4-linked fucose, while the presence of free fucose in fully methylated fucoidin suggests either that the

molecule is/.....

molecule is branched with position three of fucose units involved in the branch points, or that some of the fucose units carry two sulphate groups.

Further evidence for the presence of 1,2-glycosidic linkages in fucoidin came from the study of the products obtained by mild acetolysis of the fucoidin from Fucus vesiculosus, followed by catalytic reduction of the deacetylated fragments, when 2-O-α-L-fucopyranosyl-L-fucitol was identified in the products.[74] The presence of linkages other than the predominant 1,2-linkages was shown when Côté[75] isolated 3- and 4-O-α-L-fucopyranosyl-L-fucose in addition to the 2-linked isomer from an acetolysate of a sample of commercial fucoidin from Fucus vesiculosus.

1.2.2. Glucuronoxylofucans. Recently, studies on the extracts of Ascophyllum nodosum[76] after removal of the alginic acid has revealed the presence of three new polysaccharides the major one of which, ascophyllan, contains L-fucose, 25.3; D-xylose, 26; sodium glucuronate, 19.2; sodium sulphite, 11.9; and protein 11.8%. The other polysaccharides differ from ascophyllan in their quantitative composition. All three polysaccharides contain a firmly bound polypeptide moiety. Mild acid hydrolysis of ascophyllan yields a non-dialysable polypeptide, a mixture of free sulphated mono- and oligosaccharides based on fucose and xylose, and an acid resistant polysaccharide which contains almost all the uronic acid present in the original material and is almost devoid of fucose, xylose, and ester sulphate. These results together with those from electrophoretic, alkali, and dialysis studies on ascophyllan indicate that the macromolecule is composed of a backbone of glucuronic acid containing side chains of sulphated fucose and xylose. The isolation and tentative characterisation of 3-O-D-xylosyl-L-fucose[77] from the hydrolytic fragments indicates that

xylose and fucose are linked together in the side chains and are not present as separate side chains.

More recently Percival[78] has isolated a glucuronoxylofucan from Ascophyllum nodosum after removal of the laminarin, fucoidin, and alginic acid present. The polysaccharide contained L-fucose, 49; D-xylose, 10; sodium glucuronate, 12; sodium hydrogen sulphite, 21; and protein 4%. Partial acid hydrolysis of the free acid form of the polysaccharide led to the isolation of 3/4-D-xylosyl-L-fucose, a fucosylxylose, three oligosaccharides containing varying proportions of fucose, xylose, glucuronic acid, and ester sulphate, and a degraded polysaccharide containing fucose, 42; xylose, 12; sodium glucuronate, 29; sodium hydrogen sulphite, 12; and protein 5%. Partial hydrolysis of the polysaccharide with oxalic acid led to the isolation of 2-O-D-glucuronosyl-L-fucose. These results indicate that this polysaccharide differs from ascophyllan in that the different sugars appear to be dispersed throughout the macromolecule. However, the possible presence of 3-O-D-xylosyl-L-fucose in both polysaccharides shows that there are some similarities in the structural features of the two polysaccharides.

1.3. Polysaccharide sulphates of green algae.

The water-soluble sulphated polysaccharides of the green algae can conveniently be divided into two main groups.
(i) Polysaccharides containing D-glucuronic acid and
(ii) Polysaccharides devoid of uronic acid.

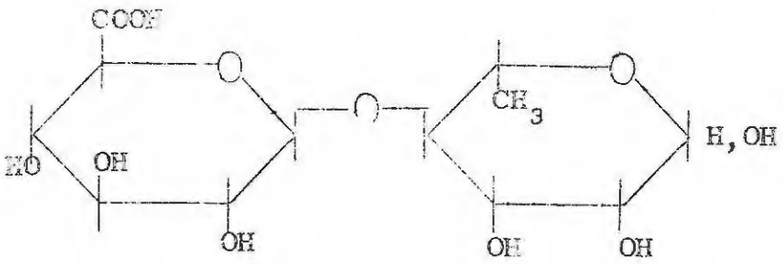
1.3.1. Polysaccharides containing D-glucuronic acid.

1.3.1.1. Ulva lactuca. Extraction of this alga with dilute sodium carbonate yielded a water soluble polysaccharide containing D-xylose, 9.4; L-rhamnose, 31; D-glucose, 7.7;

D-glucuronic acid, 19.2; and sulphate, 15.9 (as SO_4^{2-}) per cent.[79] The sulphate groups were stable to alkali and it was suggested that they were located on xylose or rhamnose residues. The small amount of periodate consumed by the polysaccharide suggested the presence of both sulphate and glycosidic linkages in positions such that α-glycol groups were essentially absent. Methylation of the polysaccharide was difficult. However, after repeated treatments with dimethyl sulphate and alkali, a product was obtained which was fractionated by extraction with chloroform. The chloroform soluble fraction contained methylated glucose, methylated rhamnose, and a small amount of sulphate. The chloroform insoluble fraction, when hydrolysed, gave 2,3,4-tri-O-methyl- and 2,3-di-O-methyl-D-xylose, 2,3,4-tri-O-methyl- and 2,3-di-O-methyl-L-rhamnose, and unmethylated rhamnose and xylose. This fraction, which had a sulphate content of thirteen per cent, was devoid of glucose or its methylated derivatives, thus suggesting that the glucose in the original polysaccharide arose from a contaminating glucan. A relatively high yield of trimethyl-L-rhamnose and of trimethyl-D-xylose suggested that these units probably arose from the non-reducing end groups of short side-chains.

Partial acid hydrolysis[80] of the cold water extract of Ulva lactuca, followed by the separation of the neutral sugars and barium uronates on cellulose and on resin, led to the isolation of an aldobiuronic acid. This acid had $[\alpha]_D$-22° and on esterification, followed by reduction with potassium borohydride, it gave a neutral disaccharide. Periodate oxidation and methylation of the methyl glycoside established that the disaccharide was 4-O-β-D-glucopyranosyl-L-rhamnose. The aldobiuronic acid was thus 4-O-β-D-glucopyranuronosyl-L-rhamnose (xiii).

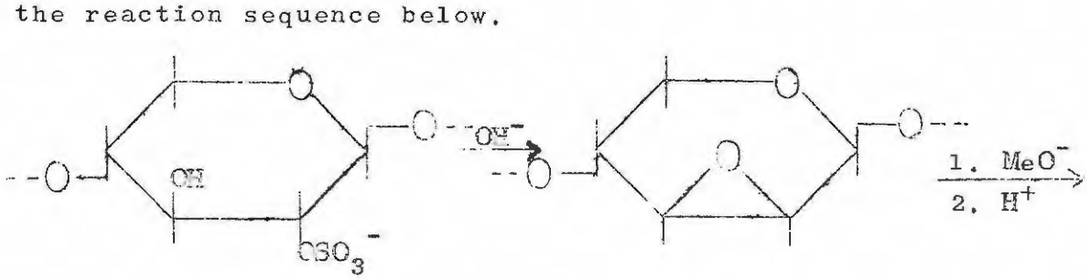
(xiii)/.....

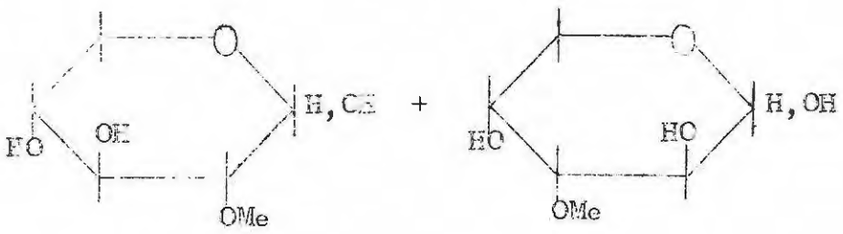


(xiii)

Fractionation studies[81] on the polysaccharide using DEAE-cellulose indicated that the polysaccharide was a single polydisperse heteropolymer.

Desulphation[81] of the polysaccharide was achieved with dry methanolic hydrogen chloride. Comparison of the periodate consumed by the polysaccharide in unbuffered solution at room temperature and in buffered solution at low temperature, before and after desulphation, established that desulphation furnished *cis*- rather than *trans*-glycol groupings. On this and infrared spectroscopic evidence, the sulphate groups were tentatively assigned to position-2 of the rhamnose residues. Comparison of the proportions of uncleaved sugars in the periodate oxidised sulphated and periodate oxidised desulphated polysaccharides showed that there was considerable loss of rhamnose in the latter and indicated the presence of some sulphated xylose. The sulphate groups on the xylose were assigned to the 2-position since alkali treatment of the polysaccharide, followed by opening with sodium methoxide of the epoxide ring so formed and then hydrolysis of the polysaccharide gave 2-*O*-methyl-D-xylose (xiv) as shown in the reaction sequence below.





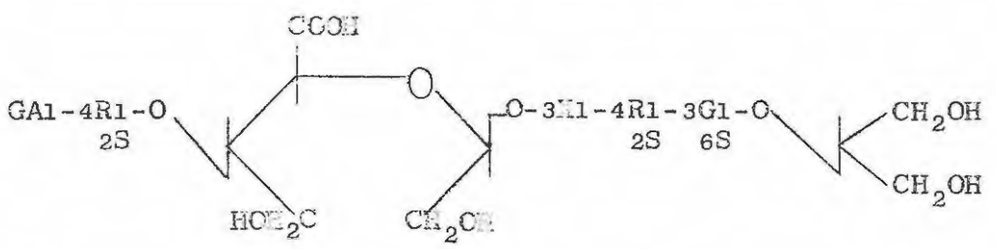
(xiv)

Methylation[82] of the desulphated reduced polysaccharide, followed by hydrolysis, led to the isolation and characterisation of 2,3,4,6-tetra-O-methylglucose, 2,3,4-tri-O-methylxylose, 2,4-di-O-methylglucose, 2,3-di-O-methylxylose, 2-O-methylrhamnose, and 2-O-methylxylose. In addition, evidence was obtained for the presence of 2,3,4-tri-, 2,3-di-, 2,4-di- and 4-O-methylrhamnose, 2,4,6-tri- and 2,3,6-tri-O-methylglucose, 2,4-di-O-methylxylose, rhamnose, and xylose. These results indicate that the macromolecule is highly branched and that 1,4- and 1,3,4-linked rhamnose, 1,4-linked xylose, and 1,3-linked glucose/glucuronic acid comprise the major structural units, and that 1,3,6-linked glucose, 1,4-linked glucose/glucuronic acid, 1,3-linked xylose, and 1,2,3-linked rhamnose are also present.

Partial hydrolysis[82] of the desulphated reduced polysaccharide resulted in the isolation of O-L-rhamnopyranosyl (1-4)-O-D-xylopyranosyl(1-3)-D-glucopyranose thus confirming the presence of 1,4-linked xylose and 1,3-linked glucose/glucuronic acid in the polysaccharide. In addition, isolation of this trisaccharide provides unequivocal proof that the three sugars are present in a single molecule. Partial hydrolysis of the original polysaccharide established 4-O-D-glucopyranuronosyl-L-rhamnose-2-sulphate as a major structural unit of the polysaccharide. In addition, smaller quantities of 3-O-D-glucopyranuronosyl-D-xylopyranose, 4-O-D-glucopyranuronosyl-D-xylopyranose/.....

nosyl-D-xylopyranose, O-D-glucopyranosyluronic acid(1-4)-L-rhamnopyranosyl(1-3)-D-glucopyranosyluronic acid (1-3)-D-xylopyranose, and 3-O-D-glucopyranosyl-D-xylopyranose were isolated. The degraded polysaccharides isolated from these partial hydrolysis studies contained the same mixture of sugars as the initial material.

Smith degradation[83] of the polysaccharide yielded glycerol, erythritol, glycollic aldehyde, 2-O-β-D-xylosyl-glycerol, 2-O-D-xylosyl-erythritol, 2-O-β-D-glucosyl-erythritol, and an acidic oligosaccharide. The isolation of the neutral constituents provides evidence for the presence of adjacent glucose, adjacent xylose, 1,4-linked glucose, and 1,4-linked xylose in the macromolecule. The results of hydrolysis, methylation, and periodate studies suggest the following possible structure (xv) for the acidic oligosaccharide.



GA = D-glucuronic acid
R = L-rhamnopyranose
G = D-glucopyranose
X = D-xylopyranose
S = Sulphate ester

(xv)

The large proportion of the acidic oligosaccharide isolated suggests that this constitutes the repeating unit with the other fragments serving as side chains. Alternatively, the adjacent 1,3- and 1,4-linked diglucose and dixylose residues may constitute the back-bone of the molecule to which are attached long acidic hetero-side-chains represented by the acidic oligosaccharide.

1.3.1.2./.....

1.3.1.2. Acrosiphonia centralis (Spongomorpha arcta).

Extraction of this alga with aqueous ammonium oxalate, followed by dialysis and freeze-drying, afforded a polysaccharide containing D-galactose, D-xylose, L-rhamnose, D-mannose, and D-glucose units together with 7.8% sulphate and 19.3% uronic acid.[84]

Periodate oxidation of the polysaccharide indicated that many of the units possess free contiguous hydroxyl groups. An acid hydrolysate of the derived oxopolysaccharide revealed the presence of small amounts of xylose and rhamnose, thus indicating the presence of either 1,3-linked units or branch points in the macromolecule. Acetylation of the polysaccharide followed by chloroform-extraction of the product gave a soluble glucose-rich acetate which was devoid of sulphate, and an insoluble acetate fraction ($[\alpha]_D$-38°). The latter was simultaneously deacetylated and methylated, and the product after hydrolysis yielded 2,3-di-(3 parts) and 2,3,4-tri-O-methyl-D-xylose(1 part); 2,3-di-(3 parts) and 2-O-methyl-L-rhamnose(5 parts). These results suggest the presence of xylose end groups, 1,4-linked xylose and rhamnose units (cf. Ulva lactuca.), and that rhamnose occurs at branch points in the molecule. In addition to the methylated neutral sugars, a series of methylated oligouronic acids was isolated. The simplest of the methylated uronic acids, i.e. the aldobiuronic acid, on esterification, followed by reduction with lithium aluminium hydride, gave a neutral disaccharide which on hydrolysis yielded 2,3,4-tri-O-methyl-D-glucose and 2-O-methyl-L-rhamnose, thus showing that the methylated aldobiuronic acid was 2,3,4-tri-O-methyl-4-O-D-glucopyranuronosyl-2-O-methyl-L-rhamnose. Partial acid hydrolysis of the polysaccharide gave, among other products, aldobi-, aldotri-, and aldotetrauronic acids. A detailed structural analysis of the aldobiuronic acid showed it to be 4-O-β-D-glucopyranuronosyl-L-rhamnose.

The aldotriunonic/.....

The aldotriuronic acid was shown to be a diglucuronosyl-O-rhamnose, while the aldotetrauronic acid fraction was shown to contain equimolar proportions of glucuronic acid and rhamnose. This evidence suggests that part of the polysaccharide consists of 1,3-linked rhamnose residues carrying D-glucuronic acid units linked at position-4.

1.3.1.3. Enteromorpha spp. Hot water extraction of Enteromorpha compressa, followed by removal of the accompanying starch, yielded a polysaccharide with the percentage composition, D-glucose, 5.7; D-xylose, 15.0; L-rhamnose, 45.0; uronic acid, 18.3 and sulphate 16.0.[85] Infrared analysis of the polysaccharide indicated the presence of axial sulphate groups. Acid hydrolysis of the polysaccharide yielded, in addition to the neutral sugars, an acidic fraction which was believed to be 4-O-β-D-glucuronosyl-L-rhamnose. Desulphation of the polysaccharide with a minimum of degradation was accomplished by treatment with methanolic hydrogen chloride. Oxidation of the polysaccharide with periodate before and after desulphation, and estimation of the molar proportions of the monosaccharides in the derived oxopolysaccharides indicated the presence of sulphated xylose and rhamnose in the original polysaccharide. Since the rhamnose appears to be glycosidically-linked through position-4, the sulphate ester must be on either position-2 or position-3. If rhamnose is assumed to be present in the polysaccharide in its stable IC conformation, in which position-2 is axial, then infrared evidence indicates the presence of rhamnose-2-sulphate.

Recently[86] acid extraction of Enteromorpha torta containing various amounts of E. compressa and E. testinales yielded a sulphated polysaccharide contaminated with 20-25% of a neutral glucan. The neutral sugar fraction of an acid hydrolysate of the polysaccharide contained 3-O-methyl-L-

rhamnose, in addition to the neutral sugars isolated from Enteromorpha compressa. Methylation of the polysaccharide mixture followed by extraction of the methylated glucan fraction with chloroform gave a residue, the methanol soluble portion of which yielded 2,3,4-tri-, 2,3- and 2,4-di-, and 2-O-methyl-L-rhamnose; L-rhamnose; 2,3-di- and 3-O-methyl-D-xylose on hydrolysis. Isolation of both rhamnose and trimethylrhamnose suggests a highly branched structure.

1.3.2. Polysaccharides devoid of uronic acid.

1.3.2.1. Cladophora rupestris. Extraction of this alga with boiling water or dilute acid, followed by precipitation with ethanol, yielded a water soluble polysaccharide, cladophoran, which contained D-galactose; L-arabinose; D-xylose; and D-glucose in the molar proportions of 2.8:3.7:1.0:0.4:0.2 together with ester sulphate (19.6%).[87] Paper electrophoresis indicated a single sulphated polysaccharide. Fractionation of cladophoran on DEAE-cellulose resulted in the separation of a purified galactose-arabinose-xylose polymer.[88] Acetylation of cladophoran followed by chloroform-extraction of the product removed all the glucose as a glucose-rich fraction which was practically sulphate free.[87] Simultaneous deacetylation and methylation of the glucose free acetate, followed by hydrolysis of the product, yielded 2,3,4,6-tetra-, 2,3,5-tri-, 2,4-di-, and 2-O-methyl-D-galactose; D-galactose; 2,4-di-, 2-O-, and 3-O-methyl-L-arabinose; L-arabinose; 2,3,4-tri- and 2,3-di-O-methyl-D-xylose; 2,4-di-, 3,4-di-, and 4-O-methyl-L-rhamnose. From this it is suggested that the polysaccharide is highly branched and contains 1,3-linked L-arabinose, D-galactose, and L-rhamnose and some 1,4-linked xylose. Oxidation of cladophoran destroyed all the xylose and about two thirds of the galactose units. This indicates the presence of 1,4-linked xylose, and xylose and galactose

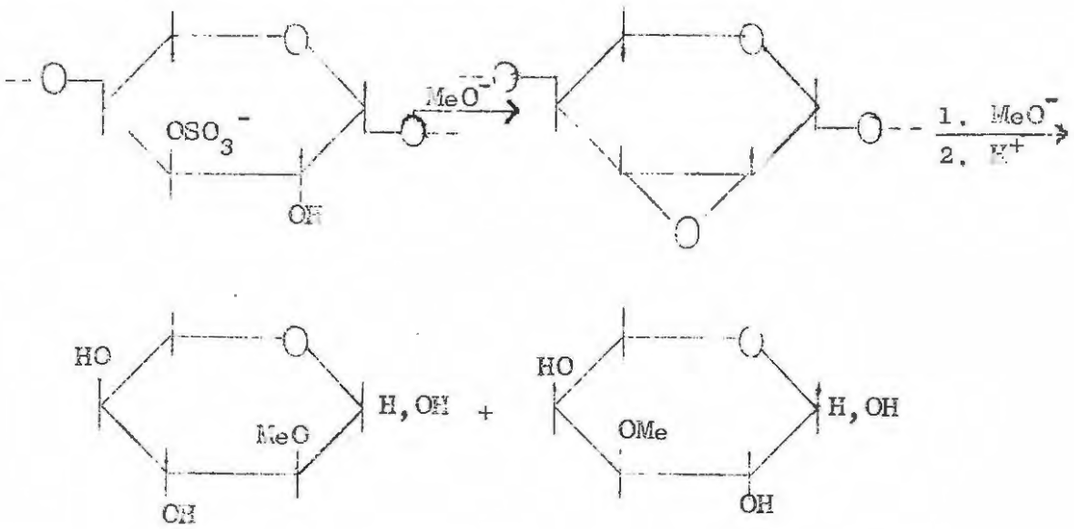
at the non-reducing/.....

at the non-reducing ends of the molecule. In an extension of this work, using the Barry degradation technique, it was confirmed that all of the xylose and much of the galactose was destroyed by one treatment with periodate.[89] After three Barry degradations, the oxopolysaccharide remaining contained arabinose, galactose, and rhamnose in the molar proportions of 1:1:0.56 together with 15.2% sulphate. Methylation of this oxopolysaccharide, followed by hydrolysis of the product, yielded 2,4-di-O-methyl rhamnose (1 part), L-rhamnose (3 parts), 2,4-di- (4 parts) and 2-O-methylarabinose (1 part), D-galactose (1 part), 2,4,6-tri- (3 parts) and 6-O-methyl-galactose (1 part). This study provides further support for a highly branched polysaccharide with an oxidation resistant core.

Partial acid hydrolysis of cladophoran led to the production of neutral oligosaccharides and a number of charged components.[88,90] Fractionation of the neutral fragments resulted in the isolation of 6-O-β-D-galactopyranosyl-D-galactose and 3-O-β-D-galactopyranosyl-D-galactose, while fractionation of the acidic components resulted in the isolation of galactose-6-sulphate, arabinose-3-sulphate, and 4-O-β-L-arabinopyranosyl-L-arabinose and 5-O-β-L-arabinopyranosyl-L-arabinofuranose with an ester sulphate linked to C-3 of the non-reducing moiety. No evidence for the presence of hetero-oligosaccharides in the partial acid hydrolysate was obtained. This suggests either that cladophoran is a mixture of sulphated homopolysaccharides, or that it contains chains, each a polymer of one particular sugar only, the chains being glycosidically linked to each other. Confirmation of the presence of ester sulphate groups on position-3 of the arabinose units was obtained by treatment of cladophoran with sodium methoxide followed by hydrolysis of the product and separation of 2-O-methyl-L-xylose. The 2-O-methyl-L-

xylose could/.....

xylose could only have arisen from 3-sulphated arabinose (xvi) as shown in the reaction sequence below.



(xvi)

Polysaccharides with similar composition also occur in Chaetomorpha linum and Ch. capillaris.

1.3.2.2. Codium fragile. Aqueous extraction of this alga, followed by removal of accompanying starch, yielded a polysaccharide containing 17% of sulphate.[91,92] A hydrolysate of the polysaccharide contained mainly D-galactose and L-arabinose with smaller amounts of D-xylose, and traces of uronic acid and L-rhamnose. Partial fractionation on diethylaminoethyl cellulose indicated the heterogeneity of the polysaccharide. Infrared analysis of two of the fractions indicated the presence of axial and of primary sulphate groups. Confirmation came when galactose-6- and -4-sulphates were isolated from a partial acid hydrolysate. In addition to the presence of sulphated galactose, alkali treatment indicated the presence of sulphated arabinose units. Periodate oxidation of the polysaccharide indicated a high proportion of 1,3 links. Direct proof of this linkage was obtained when 3-O-β-D-galactopyranosyl-D-galactose and 3-O-β-L-arabinopyranosyl-L-arabinose were isolated from a partial acid hydrolysate.

1.3.2.3. Caulerpa filiformis. Aqueous extraction of this alga, followed by removal of the accompanying starch-type glucan, yielded a polysaccharide containing galactose, mannose, xylose, and arabinose in the molar proportions of 5:2:2:1 together with ester sulphate (17.6%).[93] Traces of L-rhamnose, ribose, L-fucose, and glucose were also isolated. Partial fractionation of the free acid form was achieved with saturated barium hydroxide. The three fractions so obtained differed significantly in their contents of the various sugar residues, suggesting the presence of more than a single polysaccharide in the extract. Periodate oxidation indicates either a highly branched structure or the presence of a high proportion of 1,3 links. Evidence for the presence of some sulphate groups on the galactose units was produced by the isolation of a galactose monosulphate from a partial acid hydrolysate. No other structural information is available on this polysaccharide.

It is evident from the above review that only a few of the sulphated polysaccharides have been examined in detail. In some cases repeating structures have been proposed. However, such structures represent the gross features of the polysaccharides rather than unique structures. In several cases the structural significance of minor components of the polysaccharides have not been established. It is obvious that a closer study of the polysaccharides described above will have to be undertaken before unequivocal and unique structures can be proposed for them. In addition, the investigation of new polysaccharides is also desirable. It was only through a more detailed investigation of the agar of Gelidium amansii[19,115] and the study of the agars of several new agarophytes that the presence of 4-O-methyl-L-galactose and 6-O-methyl-D-galactose in agarose was established.

2. EXPERIMENTAL

Unless otherwise stated, concentration of solutions was carried out at 40°/20mm and specific rotations were measured in water. Paper chromatography was carried out with Whatman No 1 filter paper. The following solvent systems were used: (1) ethyl acetate - acetic acid - formic acid - water (18:3:1:4), (2) butanol - pyridine - water (9:2:2), (3) butanol - ethanol - water (40:11:9), and (4) methyl ethyl ketone saturated with water containing 1% concentrated ammonia. p-Anisidine hydrochloride,[94] periodate-benzidine,[95] aniline-diphenylamine-phosphoric acid,[96] and 20% sulphuric acid in ethanol are sprays a, b, c, and d respectively. Rgal values refer to rates of movement relative to galactose. Thin layer chromatography (t.l.c.) was carried out on glass plates coated with silica gel G containing calcium sulphate as binder, employing methyl ethyl ketone - water (85:7) as solvent. RTMG values of methylated sugars refer to the rates of travel of methylated sugars relative to tetra-O-methyl-D-galactose on thin layer plates. Gas-liquid chromatography (g.l.c.) was carried out on a Beckman GC-2A chromatograph equiped with a flame ionization detector. The stationary liquid phase, ethylene glycol succinate (14%) and polythylene glycol (0.1%), was supported on Chromosorb W (80-100 mesh) and maintained at an operating temperature of 170°. Retention times (T) are relative to that of methyl 2,3,4,6-tetra-O-methyl-β-D-glucopyranoside. Infrared spectra were recorded on a Beckman IR-8 spectrophotometer using KBr discs.

2.1. Aeodes orbitosa

2.1.1. Isolation and purification of polysaccharide.

Wet Aeodes orbitosa (6 Kg) was mixed with hot water and acetic acid added to pH 2-3. The acid caused the rapid disintegration of the weed and did not appear to degrade the polysaccharide. The mixture was heated for 0.5 h with constant stirring after the disintegration of the weed had begun, during which time the pH rose to between 6 and 7. The extract was strained through muslin and centrifuged while still hot, yielding a murky pale brown liquid. Steam was passed into the mixture for 10 minutes and the crude extract centrifuged a second time. This afforded a clear pale yellow-brown liquid. A colloidal precipitate which appeared on cooling was removed by a third centrifugation. Precipitation into ethanol (5 vols.) and washing with ethanol and finally ether afforded an off-white product (570g, 9.5% on a wet wt. basis) [Found (on material dried at 60°/0.5 mm): ash (sulphated) 16.8%]. Analysis of the ash indicated the presence of sodium as the main cation. Further purification of the polysaccharide for analysis was effected by repeated (5 times) dissolution in water, centrifugation of the solution, and precipitation in ethanol (5 vols.). Finally a solution was passed through Amberlite IR-120 resin, and the acid eluate exactly neutralised with sodium hydroxide solution, after which the solution was concentrated and precipitated in ethanol (10 vols.). The polysaccharide was collected in a centrifuge washed with ethanol, and dried, $[\alpha]_D^{18}$ +79° (c 0.53) [Found (on material dried at 60°/0.5 mm): 3,6-anhydrogalactose,[97] 0.1; ash (sulphated), 16.9; OMe, 1.9; N, 0.0; SO_4^{2-}, 25.59%; equiv. (from SO_4^{2-} detn.), 375; ν 1240 and 815 cm^{-1} (KBr disc)]. The polysaccharide failed to precipitate from solution when mixed with potassium chloride solution.

Chromatographic/.....

Chromatographic examination (solvents 1,2,3, and 4) of the hydrolysate (N sulphuric acid for 16 h at 100°) revealed spots corresponding to galactose (major, yellow-brown); 4-O-methylgalactose (trace, yellow), Rgal 1.7 (solvent 3), 2.45 (solvent 4); 6-O-methylgalactose (minor, brown), Rgal 1.85 (solvent 3), 3.3 (solvent 4); 2-O-methylgalactose (minor, orange-red), Rgal 2.87 (solvent 2), 2.1.(solvent 3), 3.9 (solvent 4); and xylose (trace, maroon), Rgal 1.78 (solvent 1) with spray a. Spray b revealed, in addition to the above monosaccharides, the presence of a very faint spot with the mobility of glycerol.

2.1.2. Separation and characterisation of the components of the polysaccharide.

Polysaccharide (70g) was hydrolysed with N sulphuric acid (400 ml) for 16 h. After neutralisation with barium carbonate the solution was deionised with Amberlite IR-120 [H^+] and Amberlite IR-4B [OH^-] resins. The neutral aqueous effluent after evaporation to a partially crystalline syrup (33.5g) was applied to a cellulose column (61 x 5.4 cm), which was eluted with butanol half saturated with water, and fractions (ca. 50 ml) collected. On the basis of paper chromatography the fractions were recombined into five fractions, which were evaporated to dryness. The following products were subsequently identified:

Fraction 1. The syrup (500 mg), chromatographically indistinguishable from glycerol, was decolourised with charcoal in water. After filtration and evaporation of the water by freeze-drying there was obtained a colourless syrup, which yielded a tri-p-nitrobenzoate, m.p. and mixed m.p. 188-190° (Kofler hot stage) with authentic glycerol tri-p-nitrobenzoate. Nunn and von Holdt[98] reported m.p. 191-193°.

Fraction 2./.....

Fraction 2. The syrup (212 mg) was decolourised with charcoal in water, filtered and evaporated to dryness, and the residue recrystallised from methanol-ethyl acetate, yielding colourless prisms, m.p. 148-149°, $[\alpha]_D^{16}$ + 84.9° (final) (c 0.53) (Found: C, 42.88; H, 7.1. Calc. for $C_7H_{14}O_6$: C, 43.3; H, 7.2%). Oldham and Bell[99] reported m.p. 147-149°, $[\alpha]_D$ + 53° → + 82.6°. This sugar moved with the mobility of authentic 2-O-methyl-D-galactose in solvent systems 1,2,3, and 4, and gave spots of the same colour as given by this sugar with spray a. When a similar paper was sprayed with triphenyltetrazolium chloride neither the sugar derived from the polysaccharide nor authentic 2-O-methyl-D-galactose were revealed, while authentic 3-O-methyl-D-galactose eluted on the same paper readily showed up (pink spot).

Demethylation[94] of the sugar (5 mg) with 48% hydrobromic acid (0.5 ml) on a boiling water-bath for 5 min, followed by chromatography of the neutralised (Ag_2CO_3) solution, revealed spots corresponding to galactose and unchanged 2-O-methyl-galactose. The sugar (50 mg), freshly distilled aniline (25 mg), and a drop of glacial acetic acid were mixed with ethanol (5 ml) and refluxed for 2 h. The anilide crystallised on cooling and, after recrystallisation from ethanol, had m.p. and mixed m.p. 164-165°[100] with authentic 2-O-methyl-N-phenyl-D-galactosylamine.

Fraction 3. The syrup (2.2g), was shown by paper chromatography (solvents 1,2,3, and 4) to consist of a mixture of 2-O-methyl and 6-O-methylgalactose together with smaller amounts of xylose and 4-O-methylgalactose. A portion (500 mg) of this fraction was separated on Whatman 3 MM paper using solvent 1. Extraction of the appropriate portion of the papers with methanol and concentration of this solution yielded a solid, which on recrystallisation (charcoal)

from ethanol gave D-xylose (19 mg), m.p. and mixed m.p. 145-146°, $[\alpha]_D^{16}$ + 18.1° (c 0.55). Percival and Wold[81] reported m.p. 144-145°, $[\alpha]_D$ + 18.3°.

Fraction 4. The syrup (730 mg) was shown by paper chromatography (solvents 1 and 3) to be a mixture of galactose (trace), xylose (trace), 4-O-methylgalactose, 2-O-methylgalactose, and 6-O-methylgalactose. The syrup was dissolved in methanol and separated on Whatman No 1 paper using solvent 4 (96 h). The portions of the papers corresponding to 4-O-methylgalactose and 6-O-methylgalactose were eluted with methanol, filtered, and evaporated to dryness. The fraction containing the 4-O-methyl sugar (10 mg) readily crystallised from methanol. It had $[\alpha]_D^{17}$ - 84.0° (final) (c 0.5), m.p. and mixed m.p. 203-206° with 4-O-methyl-L-galactose isolated from *Aeodes ulvoidea*[101] polysaccharide. The infrared spectra of the sugar derived from the polysaccharide and authentic 4-O-methyl-D-galactose were identical. The fraction containing the 6-O-methylgalactose crystallised after several days from methanol-ethyl acetate. It had $[\alpha]_D^{17}$ + 76° (c 0.6), m.p. and mixed m.p. 119-120° with authentic 6-O-methyl-D-galactose. Demethylation[94] gave galactose and unchanged 6-O-methylgalactose (paper chromatogram). Nunn and von Holdt[22] reported m.p. 122-123°, $[\alpha]_D^{17}$ + 117° → + 77.3°.

Fraction 5. A portion of this fraction (19.2g) was recrystallised from methanol (charcoal) giving galactose, m.p. and mixed m.p. 166-167°, $[\alpha]_D^{16}$ + 90.1° (c 0.71). The sugar (50 mg) was dissolved in nitric acid-water (1:1; 1 ml) and heated on a water bath at 80° for 2 h. The mucic acid crystallised on cooling and, after recrystallisation from water, had m.p. and mixed m.p. 212-213° with authentic mucic acid.

2.1.3. Ether/.....

2.1.3. Ether extraction of a hydrolysate of the polysaccharide.

In order to ensure that the glycerol was not a contaminant in the above large scale hydrolysis, and that it represents a true component of the polysaccharide, the highly purified polysaccharide (0.967g) was heated on a boiling water bath with N sulphuric acid (5 ml) for 16 h, and the neutralised hydrolysate was diluted and then extracted continuously with ether for 24 h. Removal of the ether from the dried solution (Na_2SO_4), followed by paper chromatography of the residue, showed spots having the mobility of glycerol (spray b).

2.1.4. Quantitative estimation of galactose in the polysaccharide.

The polysaccharide was hydrolysed with N sulphuric acid for 16 h. A known quantity of maltose was added to the hydrolysate which was neutralised with barium carbonate and chromatographed on Whatman No 1 in solvent 1. The strips containing galactose and maltose were cut out and macerated in water; after filtration the sugars were estimated by the Somogyi[102] micromethod [Found: galactose (calc. as $C_6H_{10}O_5$), 58%].

2.1.5. Action of alkali on polysaccharide.

Polysaccharide (3g) in water (200 ml) containing sodium borohydride[66] (0.4g) was set aside for 48 h at room temperature. Sodium hydroxide (20g) and sodium borohydride (2g) were then added, and the mixture was maintained at 80°. After 4 h a further amount of sodium borohydride (2g) was added, and, after 7 h, the solution was cooled and made slightly acid with hydrochloric acid. The mixture was dialysed against frequently changed distilled water, concentrated, and freeze-dried, yielding a white foam (2.1g), $[\alpha]_D^{17}$ + 76° (c 0.5) (Found: SO_4^{2-},

21.57;/.....

21.57; 3,6-anhydrogalactose,[97] 5.1%). Chromatography of a hydrolysate in solvents 1, 3, and 4 showed spots corresponding to galactose, 6-O-methylgalactose, 2-O-methylgalactose, xylose (trace), and 4-O-methylgalactose (trace).

2.1.6. Treatment of polysaccharide with sodium methoxide.

Polysaccharide (2g) (dried in a vacuum at 60° for 48 h) was soaked in dry methanol with occasional shaking for a further 2 days. The dried material, after rapid filtration, was added to a solution of sodium borohydride (0.3g) and sodium (6g) in dry methanol and the mixture was refluxed for 24 h. The insoluble polysaccharide, after filtration and washing with methanol, was dissolved in water (200 ml) and dialysed against frequently changed distilled water for 3 days. The solution was concentrated and freeze-dried giving a white solid (1.6g, 80%). Chromatography of a hydrolysate (solvents 1, 3, and 4) showed the presence of galactose, 2-O-methylgalactose, 4-O-methylgalactose (trace), xylose (trace), and 6-O-methylgalactose. No spot with the mobility of a dimethylhexose could be detected.

2.1.7. Desulphation by treatment with methanolic hydrogen chloride.[85]

Polysaccharide (1.99g) was shaken with 0.15M methanolic hydrogen chloride (100 ml) for 72 h at room temperature. Undissolved material was centrifuged off, and the supernatant liquid, after neutralisation with silver carbonate and concentration, gave a non-reducing syrup (582 mg). Paper chromatography of an acid hydrolysate revealed the presence of galactose, 6-O-methylgalactose, 4-O-methylgalactose (trace), xylose (trace), and 2-O-methylgalactose.

The insoluble material was washed with methanol, dissolved in water, and dialysed against distilled water.

After concentration/.....

After concentration, the polysaccharide (1.14g, 57%) was isolated by freeze-drying (Found: SO_4^{2-}, 7.65%). The peak at 815 cm^{-1} recorded in the infrared spectrum of the parent polysaccharide was absent, and the peak at 1240 cm^{-1} was much smaller.

Decreasing the strength of the methanolic hydrogen chloride to 0.09 M resulted, after a single treatment (48 h), in a 83% yield of polysaccharide, $[\alpha]_D^{18}$ + 77° (c 0.64) (Found: SO_4^{2-}, 24.8%). Further shaking with 0.15 M methanolic hydrogen chloride for 114 h gave a 36% yield of polysaccharide, $[\alpha]_D^{18}$ + 94° (c 0.34) (Found: SO_4^{2-}, 3.45%). Paper chromatograms of both an acid hydrolysate of the polysaccahride and an acid hydrolysate of the non-reducing, methanol soluble syrup revealed the presence of galactose, xylose, 2-O-methylgalactose, 4-O-methylgalactose, and 6-O-methylgalactose.

In a third experiment polysaccharide was shaken with 0.15 M methanolic hydrogen chloride for 48 h at room temperature and isolated (yield, 81%) as above (Found: SO_4^{2-}, 16.8%). After a further 48 h treatment, the yield was reduced to 37% (Found: SO_4^{2-}, 6.3%).

2.1.8. Periodate oxidation of polysaccharides.

Polysaccharide (29.5 mg), desulphated polysaccharide (SO_4^{2-}, 6.3%; 24.7 mg), and desulphated polysaccharide (SO_4^{2-}, 3.45%; 24.2 mg) were dissolved separately in water (5 ml each) and 0.288 M sodium metaperiodate (5 ml) was added to each solution. The solutions were set aside at room temperature in the dark, and at intervals aliquots (0.10 ml) were removed and the reduction of periodate measured[103] (Table 2).

2.1.9. Reduction of periodate oxidised polysaccharide.[104]

(a)- Polysaccharide (3g) and sodium metaperiodate (21.4g)

in water/.....

in water (500 ml) was kept in the dark at room temperature for 36 h, when reduction of periodate was complete. After removal of excess of periodate with ethylene glycol, sodium borohydride (5g) was added and the mixture set aside overnight. The solution was dialysed (4 days) against frequently changed distilled water, and concentrated, and the polysaccharide alcohol (2.55g) was isolated by freeze-drying. The polysaccharide alcohol (500 mg) was heated with N sulphuric acid on a boiling water bath for 12 h, and the neutralised ($BaCO_3$) hydrolysate was filtered, and concentrated by freeze-drying to a syrup. Paper chromatography revealed the presence of galactose, 6-O-methylgalactose, 2-O-methylgalactose, and 4-O-methylgalactose (trace) with spray a, whereas chromatograms treated with spray b revealed spots having the mobilities of glycol aldehyde and glycerol (trace), in addition to the above four sugars. The syrup was diluted with water (10 ml) and shaken with Amberlite IRA-400 [OH^-] resin until the solution gave a negative Molisch test. The treatment with strong base resin was done in order to specifically remove the 2-O-methyl-D-galactose (strong base resins react with sugars) which could not be separated from threitol (which was suspected to be present) chromatographically. The solution was then filtered, and concentrated to a colourless syrup by freeze-drying. Paper chromatography of an aliquot revealed spots having the mobilities of glycerol (trace) and threitol (spray b); similar chromatograms failed to reveal any spots with spray a. T.l.c. of a second aliquot (ethyl acetate-n-propanol-water, 2:7:1) revealed spots (alkaline permanganate spray) having the mobilities of glycerol and threitol.

(b)- Desulphated, degraded polysaccharide (SO_4^{2-}, 6.3%; 0.32g) and sodium metaperiodate (2.14g) in water (30 ml) was allowed to stand for 96 h and then treated as in the previous case.

The oxidation/.....

The oxidation in this case took much longer. Paper chromatograms of a neutralised hydrolysate of the oxopolysaccharide (recovered, 0.1g) revealed the presence of galactose, 2-O-methylgalactose, 6-O-methylgalactose, and 4-O-methylgalactose. The oxopolysaccharide, after reduction with borohydride and subsequent treatment as in the above case, yielded a solution, which exhibited a single spot having the mobility of threitol on chromatography.

2.1.10. Methylation of polysaccharide.

Polysaccharide (24.7g) in water (500 ml) was treated slowly and simultaneously with dimethyl sulphate (150 ml) and sodium hydroxide solution (30% w/v; 450 ml) with vigorous stirring during 7 h after which the mixture was stirred for a further 17 h. This was repeated four times, when the final solution was dialysed against running water (2 weeks), and the polysaccharide was isolated by freeze-drying. Methylation was incomplete as shown by the presence of a large proportion of galactose on chromatography of a neutralised hydrolysate. After a further fourteen additions of the above reagents, a hydrolysate was found to contain only a minute trace of galactose. The product was isolated by concentration of the dialysed solution followed by freeze-drying (yield, 11.2g) (Found OMe 19.8; SO_4^{2-}, 16.5%).

2.1.11. Hydrolysis of methylated polysaccharide and separation of the products.

Methylated polysaccharide (11.0g) was hydrolysed on a boiling-water bath with N sulphuric acid (100 ml) for 16 h. The hydrolysate was neutralised with barium carbonate, centrifuged, and concentrated to a brown syrup, which was applied to a cellulose column (60 x 5.4 cm), and eluted with butanol - water (95:5). On the basis of paper chromatography

appropriate/.....

appropriate fractions were recombined into the following seven fractions.

Fraction 1 contained traces of tetra-O-methylgalactose (paper chromatography), together with degradation products arising from the hydrolysis.

Fraction 2, a syrup, chromatographically identical with tetra-O-methylgalactose, gave a crystalline anilide, m.p. and mixed m.p. 188-189° with 2,3,4,6-tetra-O-methyl-N-phenyl-D-galactosylamine. Clingman and Nunn[55] reported m.p. 189-190°,

Fraction 3, a syrup, shown by t.l.c. (spray c) to be a mixture of 2,4,6- and 2,3,6-tri-O-methylgalactose, together with a trace of the 2,3,4-isomer, was applied, in the minimum volume of water, to a charcoal-Celite column (60 x 5.4 cm). Linear gradient elution was effected with aqueous methyl ethyl ketone, initially at 1% and finally at 5% of the latter component over 10 litres of eluant. On the basis of t.l.c., the fractions were recombined into three fractions.

Fraction 3a, a syrup $[\alpha]_D^{18}$ + 91.5° (c 0.5), chromatographically (t.l.c., spray c) identical with 2,4,6-tri-O-methylgalactose, yielded an anilide which, after recrystallisation from ethanol - ethyl acetate, had m.p. and mixed m.p. 171-172° with 2,4,6-tri-O-methyl-N-phenyl-D-galactosylamine. Clingman and Nunn[55] reported 170.5-171.5°. The osazone,[49] on recrystallisation from aqueous ethanol, had m.p. 154-155°.

Fraction 3b, a syrup, was shown by chromatography (t.l.c., spray c) to be a mixture of 2,3,6- and 2,4,6-tri-O-methylgalactose.

Fraction 3c, a syrup $[\alpha]_D^{18}$ + 90° (c 0.37), was chromatographically identical with 2,3,6-tri-O-methyl-D-

galactose./.....

galactose. The derived 2,3,6-tri-O-methyl-D-γ-galactono-lactone[105] had m.p. 97-99°.

Fraction 4 contained a mixture of 2,6- and 4,6-di-O-methyl-D-galactose, which was separated on a cellulose column (60 x 5.4 cm) by elution with solvent 1. The 2,6-di-O-methyl-D-galactose, after crystallisation from ethyl acetate, had $[\alpha]_D^{18}$ + 86° (c 0.5), m.p. and mixed m.p. 129-130°[55] with an authentic sample.

Fraction 5 contained 4,6-di-O-methyl-D-galactose which, after several recrystallisations from ethyl acetate, had m.p. 136-138°, $[\alpha]_D^{16}$ + 117.6° (5 min) → + 73.8° (c 0.87) (Found: C, 46.1; H, 7.9. Calc. for $C_8H_{16}O_6$, C, 46.1: H, 7.8%). Dolan and Recs[49] reported m.p. 146-147°, $[\alpha]_D$ + 120° (5 min) → + 74°, whereas Bell[106] reported m.p. 131-133° for 4,6-di-O-methyl-D-galactose. Demethylation[94], followed by paper chromatography, showed the presence of galactose. It readily gave a phenylosazone on treatment with redistilled phenylhydrazine in the presence of sodium metabisulphite, m.p. and mixed m.p. 154-155° with the osazone derived from fraction 3a. Dolan and Rees[49] reported m.p. 155° for 4,6-di-O-methyl-D-galactosazone. This sugar, when treated with aniline and a trace of glacial acetic acid, gave a crystalline anilide which, after recrystallisation from ethanol, had m.p. 149-150°. Hirst and Jones[107] reported m.p. 207° for 4,6-di-O-methyl-N-phenyl-D-galactosylamine.

Fraction 6 crystallised when triturated with methanol, and was a mixture of 4,6-di-O-methylgalactose and 4-O-methylgalactose (paper chromatography).

Fraction 7 crystallised from ethanol or ethanol-water, yielding prisms of 4-O-methyl-D-galactose, m.p. 202-209°, $[\alpha]_D^{20}$ (5 mins) + 63.5° → + 82.5° (c 1.26). The anilide

crystallised from/.....

crystallised from ethanol in needles, m.p. 166-167°. Hirst and Jones[108] reported m.p. 207° for 4-O-methyl-D-galactose, and 168° for 4-O-methyl-N-phenyl-D-galactosylamine.

2.1.12. Methylation of desulphated polysaccharide.

Polysaccharide (4.8g; SO_4^{2-}, 6.3%) was dissolved in dimethyl sulphoxide (60 ml) with gentle heating, and then dimethylformamide (60 ml) was added. After cooling in ice for 0.5 h, barium hydroxide octahydrate (60g) was added with stirring. This was followed, after a further 0.5 h, by dimethyl sulphate (10 ml). Further additions of dimethyl sulphate (10 ml) were made after 1, 1.5, 2, and 2.5 h. The mixture was stirred in a closed system for 72 h at room temperature, and concentrated ammonia solution (25 ml) was then added, followed by vigorous shaking for 0.5 h to decompose the excess of dimethyl sulphate. After the addition of water (300 ml), the mixture was dialysed against running tap water for two weeks. The solution was centrifuged and extracted with chloroform (6 x 400 ml). Evaporation of the combined chloroform solutions yielded a gum (2.4g) that was incompletely methylated as revealed by the infrared spectrum. The aqueous solution containing the chloroform insoluble material, on concentration and freeze-drying, yielded a partially methylated polysaccharide (2.15g). This fraction was not further investigated.

The chloroform soluble gum (2.4g) was dissolved in dimethylformamide (15 ml), methyl iodide (20 ml) and silver oxide (20g) added, and the mixture shaken for 48 h. After a further three treatments with methyl iodide (15 ml) and silver oxide (15g) the methylated polysaccharide (1.25g) was isolated (Found: SO_4^{2-}, 1.65; OMe, 35.0%). The infrared spectrum of the product showed a very small hydroxyl peak. Further treatment with Purdie's reagents[113] failed to increase

the methoxyl/.....

the methoxyl content.

2.1.13. Hydrolysis of desulphated methylated polysaccharide and separation of the products.

Hydrolysis of the methylated polysaccharide (OMe, 35%; 1.2g) with N sulphuric acid for 16 h, followed by neutralisation with barium carbonate, filtration, and evaporation, afforded a syrup (0.7g), chromatographic examination of which revealed the presence of tetra-O-methylgalactose, two tri-O-methylgalactoses, and two di-O-methylgalactoses. The hydrolysate appeared to be devoid of monomethylgalactoses and unmethylated sugars.

A portion of the hydrolysate (500 mg) was separated on Whatman No 20 paper by elution with solvent 2 for 36 h, and the fractions were examined as follows:

Tetra-O-methyl fraction.

The syrup (25 mg) was chromatographically (t.l.c.) identical with 2,3,4,6-tetra-O-methylgalactose. The derived anilide,[55] after recrystallisation from ethanol, had m.p. and mixed m.p. 188-189° with 2,3,4,6-tetra-O-methyl-N-phenyl-D-galactosylamine.

Tri-O-methyl fraction.

The syrup (250 mg) was shown by chromatography (t.l.c., spray c) to be a mixture of 2,4,6- and 2,3,6-tri-O-methylgalactose. This syrup was separated into two fractions on a charcoal-Colite column (60 x 5.4 cm) by gradient elution with aqueous methyl ethyl ketone (1 to 5%) over ten litres of eluant. The major fraction, $[\alpha]_D^{18}$ + 90° (c 0.47), on treatment with aniline yielded an anilide which, after recrystallisation from ethanol-ethyl acetate, had m.p. and mixed m.p. 171-172° with 2,4,6-tri-O-methyl-N-phenyl-D-galactosylamine.[55]/.....

amine.[55] The minor fraction gave a single spot on chromatography (t.l.c., spray c), which was identical both in colour (grey) and mobility with that of authentic 2,3,6-tri-O-methylgalactose, but different from 2,4,6- and 2,3,4-tri-O-methylgalactoses eluted on the same plate.

Di-O-methyl fraction.

The syrup (50 mg) was shown by chromatography to be a mixture of approximately equal parts of 2,6- and 4,6-di-O-methylgalactoses.

2.1.14. Autohydrolysis of polysaccharide.

The polysaccharide in aqueous solution (5%; 300 ml) was converted into the free acid form (Amberlite IR-120 [H^+] resin) and then dialysed against distilled water (1 l). After three days, the dialysate was neutralised ($BaCO_3$), filtered, and concentrated to a brown syrup (0.9g). Paper chromatographic examination of the syrup revealed the presence of high molecular weight material only.

2.1.15. Partial hydrolysis of polysaccharide.

Polysaccharide (10g) in N sulphuric acid (200 ml) was heated on a boiling water bath of 1 h. Neutralisation of the hydrolysate ($BaCO_3$), followed by deionisation (Amberlite IR-120 [H^+] and IR-4B [OH^-] resins), and concentration yielded a brown syrup (3g) which was separated on Whatman 3 MM paper (solvent 1 for 48 h). Extraction of the appropriate portions of the paper with water, followed by concentration, afforded two pure oligosaccharides.

Oligosaccharide 1 was obtained as an amorphous, hygroscopic solid (105 mg), and had $[\alpha]_D^{17}$ + 60.0°[+] (c 0.65), DP^{109} 2.2, and gave galactose on hydrolysis. It gave a grey-green

colour with/.....

[+] cloudy solution

colour with spray c and a red colour with triphenyltetrazolium spray. The sugar (100 mg) was thoroughly dried, dissolved in distilled dimethylformamide (10 ml) and cooled to 0°. Freshly distilled methyl iodide (10 ml) and dry silver oxide (10g) were added and the mixture shaken in the dark at 0° for 6 h and then for 42 h at room temperature. The product was filtered and the silver salts washed with chloroform. The filtrate and washings were evaporated to a syrup and traces of dimethylformamide removed under high vacuum. T.l.c. of the product indicated (spray d) that methylation was incomplete. Hence the product was given three treatments with Purdie's reagents,[113] after which, methylation was found to be complete (t.l.c. and infrared spectrum). The methylated disaccharide was hydrolysed with N sulphuric acid, neutralised, and concentrated to a syrup which was separated on Whatman No 20 paper with solvent 2 for 36 h. The following sugars were subsequently identified:

2,3,4,6-Tetra-O-methyl-D-galactose. A syrup (20 mg), $[\alpha]_D^{17}$ + 90.5° (c 0.5). The derived anilide had m.p. and mixed m.p. 188-190° with authentic 2,3,4,6-tetra-O-methyl-N-phenyl-D-galactosylamine.[55]

2,4,6-Tri-O-methyl-D-galactose. A syrup (24 mg), $[\alpha]_D^{17}$ + 92.5° (c 0.55). The derived anilide had m.p. and mixed m.p. 170-171° with authentic 2,4,6-tri-O-methyl-N-phenyl-D-galactosylamine.[55] This oligosaccharide is therefore 3-O-β-D-galactopyranosyl-D-galactose.

Oligosaccharide 2 (120 mg) readily crystallised from aqueous ethanol and, after recrystallisation from the same solvent, had m.p. 205-208°, $[\alpha]_D^{17}$ + 73.2° (c 0.41). It gave a blue colour with spray c and a red colour with triphenyltetra-

zolium/.....

zolium spray. Chromatography of a partial acid hydrolysate (N sulphuric acid for 10 min) revealed the presence of galactose and unchanged material. A sample (10 mg) was dissolved in dimethylformamide (2 ml) and methylated with methyl iodide (2 ml) and silver oxide (2g) as for the above disaccharide. T.l.c. of the product indicated (spray d) that methylation was incomplete. After three treatments with Purdie's reagents,[113] the methylated product was refluxed with 2% methanolic hydrogen chloride for 12 h and the derived methylglycosides examined by g.l.c. Peaks corresponding to 2,3,4,6-tetra-O-methylgalactose (T 2.00) and 2,3,6-tri-O-methylgalactose (T 3.7, 4.68, 5.26, and 5.84) were observed. In addition, a small peak due to 2,3,4-tri-O-methylgalactose (T 9.70) was observed. The molar ratio of 2,3,4,6-tetra-O-methylgalactose : 2,3,6-tri-O-methylgalactose was 1 : 1.3. This oligosaccharide is therefore 4-O-β-D-galactopyranosyl-D-galactose.

2.1.16. Examination of polysaccharide in the ultracentrifuge.

The polysaccharide was examined at a concentration of 0.5% in 0.1M phosphate buffer (pH 6.8) at an ultracentrifuge speed of 56,100 r.p.m.

2.2. Phyllymenia cornea

2.2.1. Extraction and purification of polysaccharide.

Wet Phyllymenia cornea was mixed with hot water and acetic acid added to pH 3. The mixture was heated (0.75 h) with constant stirring until the weed had disintegrated. The solution was strained through muslin and centrifuged while still hot, yielding a clear white mucilage. The residual weed was extracted a second and a third time, and the combined mucilages were set aside for 24 h at 4°. A colloidal precipitate which appeared was removed by a second centrifugation Precipitation into ethanol (5 vols.) afforded a fibrous, white product which was collected, washed with ether, and dried. Purification of the polysaccharide was effected by dissolution in water, centrifugation of the solution, and precipitation into ethanol (5 vols.). The polysaccharide was collected, washed with ether, and dried in vacuo at 45°. The properties of the polysaccharides isolated from three samples of Phyllymenia are compared in Table 1.

Chromatographic examination (solvents 1, 2, 3, and 4) of the neutralised acid hydrolysate of each of the polysaccharides revealed the presence of galactose (major), 2-O-methylgalactose (major), 6-O-methylgalactose, 4-O-methylgalactose (trace), and xylose (trace) with sprays a and b. The polysaccharide failed to precipitate from solution when mixed with potassium chloride solution.

2.2.2. Separation and characterisation of the components of the polysaccharide.

Polysaccharide (16g; $[\alpha]_D^{16}$ + 81.2°; SO_4^{2-}, 18.09%) was heated on a boiling water bath with N sulphuric acid (75 ml) for 16 h. The hydrolysate was neutralised with barium carbonate, deionised with Amberlite IR-120 [H^+] and Amberlite IR-4B [OH^-] resins, and evaporated to a thick, mobile syrup (9.5g) which was applied to a cellulose column (47 x 5.4 cm). The column was eluted with butanol-water (95:5), and fractions

Table 1.....

Table 1

Polysaccharides extracted from Phyllymenia cornea

Date of collection	15-7-1966	22-8-1966	15-1-1967 to 26-1-1967
Place	Palmiet	Kowie Point	Palmiet
Weight of Phyllymenia	320	200	2,400 g
Weight of polysaccharide	32	10	240 g
$[\alpha]_D^{16}$	+ 81.2° (c 0.64)	+ 81.1° (c 0.74)	+ 63.3° (c 0.49)
N	0.00	0.00	0.48%
OCH_3	5.09	4.30	4.98%
SO_4^{2-}	18.09	19.28	19.62%
$NaSO_3^-$ [A]	19.41	20.68	21.05%
3,6-anhydro-galactose[97]	0.52	0.40	0.56%
galactose [B]	43.40	45.22	42.20%
monomethylgalactoses [C]	28.88	24.4	28.27%
sulphated ash	8.24	14.78	15.91%
Molar ratio of A : B : C	19:27:16	20:28:14	20:26:16

(ca. 50 ml) collected. On the basis of paper chromatography the fractions were recombined into four fractions, which were evaporated to dryness. The following products were subsequently identified:

Fraction I, a syrup (0.34g), contained the degradation products of the hydrolysis.

Fraction II, a syrup (2.3g), was shown by paper chromatography (solvent 3) to be a mixture of 2-O-methylgalactose (major sugar, Rgal 2.1) and 6-O-methylgalactose (Rgal 1.85). The syrup was decolourised with charcoal in water, filtered, and evaporated to dryness, and the residue dissolved in ethanol-ethyl acetate, whereupon the 2-O-methylgalactose readily crystallised. After recrystallisation from the same solvent the crystals had m.p. and mixed m.p. 146-148° with authentic 2-O-methyl-D-galactose, $[\alpha]_D^{17}$ + 56.6° (2 min) → + 86.6° (c 0.6). Oldham and Bell[99] reported m.p. 147-149°, $[\alpha]_D$ + 53° → + 82.6°. The infrared spectrum of this sugar was identical with that of authentic 2-O-methyl-D-galactose, but different from that of 6-O-methyl and 4-O-methylgalactose.

Fraction III, a syrup (0.73g), was shown by paper chromatography (solvents 1, 3, and 4) to contain 6-O-methylgalactose (major sugar), xylose, 2-O-methylgalactose, and 4-O-methylgalactose. The syrup was decolourised with charcoal in water, filtered, and evaporated to dryness, and the residue dissolved in methanol. After a week, needle crystals (5 mg), m.p. 202-207°, $[\alpha]_D^{16}$ - 53.4° (2 min) → - 83.5° (c 0.34) had deposited. This sugar moved with the mobility of authentic 4-O-methylgalactose in solvents 1, 2, 3, and 4. Demethylation[94] with hydrobromic acid, followed by paper chromatography, revealed the presence of galactose and unchanged material. This sugar showed no depression in m.p. when mixed with 4-O-methyl-L-galactose isolated from the hydrolysate of the sulphated

polysaccharide/.....

polysaccharide extracted from Aeodes ulvoidea.[101] However, when mixed with authentic 4-O-methyl-D-galactose the m.p. was depressed to 186-192°. The infrared spectra of the D and L sugars were identical.

Fraction IV, a crystalline solid (2.1g), was crystallised from ethanol and, after several recrystallisations from the same solvent, had $[\alpha]_D^{16}$ + 80° (c 1.0), m.p. and mixed m.p. 166-167° with authentic D-galactose. Oxidation with nitric acid - water (1:1) yielded mucic acid, m.p. and mixed m.p. 212-213°.

2.2.3. Action of alkali on polysaccharide.

Polysaccharide (1.57g; $[\alpha]_D^{16}$ + 81.1°; SO_4^{2-}, 19.28%) in water (200 ml) containing sodium borohydride[66] (0.5g) was set aside for 48 h at room temperature, to prevent end group degradation by the alkali. Sodium hydroxide (20g) and sodium borohydride (1.5g) were added to the resulting mixture, and the solution was heated at 80° for 9 h. Further additions of potassium borohydride (0.5g) were made every hour. After dialysis against frequently changed distilled water, the derived solution was concentrated and freeze-dried to a white foam (1.4g; 89%). It had $[\alpha]_D^{16}$ + 43.5° (c 0.83) [Found: 3,6-anhydrogalactose,[97] 0.67; SO_4^{2-}, 17.67%]. Chromatography of an acid hydrolysate (solvents 3 and 4) revealed the presence of galactose, 2-O-methylgalactose, 4-O-methylgalactose (trace), 6-O-methylgalactose (trace), and xylose (trace).

2.2.4. Desulphation of polysaccharide.[85]

Polysaccharide (1g; $[\alpha]_D^{16}$ + 81.1°; SO_4^{2-}, 19.28%) was shaken with 0.15M methanolic hydrogen chloride (75 ml) at room temperature for 48 h. The undissolved material was filtered off and washed with methanol (3 x 30 ml). The filtrate

and washings/.....

and washings were combined, neutralised with silver carbonate, and concentrated to a non-reducing syrup (350 mg). Paper chromatography of an acid hydrolysate of the syrup showed the presence of galactose (major sugar), 6-O-methylgalactose (minor sugar), and traces of 2-O- and 4-O-methylgalactose.

The insoluble material was dissolved in water (100 ml) and dialysed (3 days) against distilled water. After concentration, the polysaccharide (0.69g) was isolated by freeze-drying. It had $[\alpha]_D^{17}$ + 90° (c 0.8) [Found: SO_4^{2-}, 0.0%]. Paper chromatography of an acid hydrolysate revealed the presence of galactose, 2-O-methylgalactose, and minute traces of 4-O- and 6-O-methylgalactose.

In another experiment polysaccharide (1g; $[\alpha]_D^{16}$ + 81.1°; SO_4^{2-}, 19.28%) was shaken with 0.1M methanolic hydrogen chloride (75 ml) for 48 h to give a 74% yield of polysaccharide $[\alpha]_D^{17}$ + 95° (c 0.61) [Found: SO_4^{2-}, 8%].

In a third experiment polysaccharide (1g; $[\alpha]_D^{16}$ + 81.1°; SO_4^{2-}, 19.28%), after being shaken for 72 h with 0.1M methanolic hydrogen chloride, gave a 70% yield of polysaccharide [Found: SO_4^{2-}, 4.7%]. Paper chromatography of an acid hydrolysate of the polysaccharide revealed the presence of galactose, 2-O-methylgalactose, and traces of 4-O- and 6-O-methylgalactose, while paper chromatography of a hydrolysate of the derived methyl glycosides showed the presence of galactose, 6-O-methylgalactose, and minute traces of 2-O- and 4-O-methylgalactoses.

In a fourth experiment polysaccharide ($[\alpha]_D^{16}$ + 63.3°; SO_4^{2-}, 19.62%) gave a 67% yield of polysaccharide after a single (48 h) treatment with 0.15M methanolic hydrogen chloride [Found: SO_4^{2-}, 12.3%].

2.2.5. Periodate oxidation of polysaccharides.

Polysaccharide (28.9 mg; $[\alpha]_D^{16}$ + 81.1°; SO_4^{2-}, 19.28%),

desulphated/.....

desulphated polysaccharide (29 mg; $[\alpha]_D^{17}$ + 90°; SO_4^{2-}, 0%), and partially desulphated polysaccharide (25 mg; SO_4^{2-}, 4.7%) were dissolved separately in water (5 ml each) and 0.298M sodium metaperiodate (5 ml) added to each solution. The solutions were set aside at room temperature in the dark, and at intervals aliquots (0.10 ml) were withdrawn and the reduction of periodate measured spectrophotometrically[103] (Table 3).

2.2.6. Methylation of polysaccharide.

Polysaccharide (26.2g; $[\alpha]_D^{16}$ + 63.3°; SO_4^{2-}, 19.62%) in water (500 ml) was treated slowly and simultaneously (under an atmosphere of nitrogen) with dimethyl sulphate (150 ml) and sodium hydroxide solution (30% w/v; 450 ml) with vigorous stirring during 7 h. The mixture was then stirred for a further 17 h. After repeating the methylation procedure a further six times, the mixture was dialysed against running tap water (10 days), concentrated to ca. 500 ml, and the addition of the above reagents repeated a further six times. The polysaccharide (24g) was isolated from the mixture by freeze-drying after dialysis and concentration. Thin layer chromatography (spray c) of a neutralised acid hydrolysate revealed the presence of galactose together with a large number of its methyl ethers. The methylation procedure was therefore repeated a further five times on a portion (16g) of the partially methylated material. The polysaccharide (15.8g) was isolated as above (Found: OMe, 17.1; N, 0.0; SO_4^{2-}, 16.38%). T.l.c. of a hydrolysate revealed the presence of galactose, 4-O-methyl, 2-O-methyl, 2,6-di-O-methyl, 4,6-di-O-methyl, 2,3,6-tri-O-methyl, and 2,4,6-tri-O-methylgalactose together with minute traces of 2,3,4-tri-O-methyl and 2,3,4,6-tetra-O-methylgalactose. The partially methylated polymer (2.3g) was mixed with dry dimethyl sulphoxide

and vigorously/.....

and vigorously stirred at room temperature for 2 h to disperse the polysaccharide. Silver oxide (30g) and methyl iodide (60 ml) were added and the mixture stirred for 24 h at room temperature. Two further additions of Purdie's reagents[113] were made and each time the mixture was stirred for 24 h at 50°. A large volume of water was added to precipitate the salts and the mixture centrifuged, dialysed, and the polysaccharide (1.1g) isolated by freeze-drying (Found: OMe, 24.7; N, 0.27; SO_4^{2-}, 6.7%).

2.2.7. Hydrolysis of methylated polysaccharide and separation of the products.

Methylated polysaccharide (1g) and N sulphuric acid (15 ml) were heated on a boiling water bath for 16 h. The hydrolysate was neutralised with barium carbonate, filtered, and concentrated to a brown syrup (0.9g), which was applied to a cellulose column (45 x 5.4 cm) and the methylated sugars eluted with solvent 1. Each fraction (ca. 60 ml) was analysed for sugars by t.l.c. (spray c), and like fractions were combined, evaporated to dryness under reduced pressure, and extracted with ethyl acetate or methanol. The extract, after removal of the solvent, was placed under high vacuum to remove the last traces of acetic acid and was then weighed. The following products were subsequently identified:

Tubes 1-5 contained the degradation products (86 mg) arising from the hydrolysis.

Tubes 6 and 7 contained 2,3,4,6-tetra-O-methylgalactose (28 mg). The syrup was decolourised with charcoal and converted into a crystalline anilide which after recrystallisation from ethanol had m.p. and mixed m.p. 188-189° with authentic 2,3,4,6-tetra-O-methyl-N-phenyl-D-galactosylamine. Clingman and Nunn[55] reported m.p. 189-190°.

Tube 8/.....

Tube 8 gave a syrup (30 mg), $[\alpha]_D^{17}$ + 89° (c 0.56), chromatographically (RTMG, 0.86; grey spot; spray c) identical with 2,3,6-tri-O-methylgalactose. The syrup (30 mg) was oxidised with bromine water (96 h), and, after removal of bromine by aeration, the solution was neutralised with silver carbonate and filtered, and silver ion precipitated with hydrogen sulphide. The filtrate from this treatment was evaporated to dryness *in vacuo*, and the residue extracted with dry ether; colourless needles, m.p. and mixed m.p. 99-100° with authentic 2,3,6-tri-O-methyl-D-γ-galactonolactone,[105] were obtained on concentration of the extract.

Tubes 9 and 10 contained a mixture (102 mg) of 2,3,6-tri-O-methylgalactose (RTMG, 0.86; grey spot; spray c) and 2,4,6-tri-O-methylgalactose (RTMG, 0.66; blue-green spot; spray c). The 2,3,6-isomer constituted approximately 80% of the mixture.

Tubes 11-14 afforded a syrup (67 mg) which was shown by paper and t.l.c. to be a mixture of 2,3,6-tri-, 2,4,6-tri-, and 2,6-di-O-methylgalactose together with a trace of 2,3,4-tri-O-methylgalactose and an unidentified sugar (RTMG, 0.7; Whatman No 1; carmine spot; spray a).

Tube 16 afforded a syrup (51 mg; RTMG, 0.53; grey spot; spray c) which crystallised from ethyl acetate and, after recrystallisation from the same solvent, had $[\alpha]_D^{16}$ + 47.1° (2 min) → + 85.8° (c 0.7), m.p. and mixed m.p. 128-129° with authentic 2,6-di-O-methyl-D-galactose. Clingman and Nunn[55] reported $[\alpha]_D$ + 45° → + 88°, m.p. 128.5-130° for 2,6-di-O-methyl-D-galactose.

Tubes 17 and 18 contained a mixture (103 mg) of 2,6-di-O-methylgalactose (RTMG, 0.53) and 4,6-di-O-methylgalactose (RTMG, 0.33; blue spot; spray c). The 4,6-di-O-methyl ether which constituted approximately 70% of the mixture readily

crystallised when/.....

crystallised when the syrup was mixed with ethyl acetate.

Tubes 19-24 afforded a syrup (103 mg) which crystallised (charcoal) from ethyl acetate and had m.p. 140-142° and, after two recrystallisations from the same solvent, had m.p. 146-147°, $[\alpha]_D^{16}$ + 131.2° (2 min) → + 72° (c 0.64). The mixed m.p. with a sample of 4,6-di-O-methyl-D-galactose isolated from methylated aeodan was 142°. The infrared spectra of the two sugars were identical. The 4,6-di-O-methyl-D-galactose from Phyllymenia polysaccharide readily gave an osazone, m.p. and mixed m.p. 154-155° with 4,6-di-O-methyl-D-galactosazone prepared from the sugar isolated from aeodan. Dolan and Rees reported[49] m.p. 146-147°, $[\alpha]_D$ + 120° (5 min) → + 74° for 4,6-di-O-methyl-D-galactose and m.p. 155° for 4,6-di-O-methyl-D-galactosazone.

Tubes 25-35 contained no carbohydrate.

Tubes 36-41 contained 2-O-methylgalactose (58 mg; Rgal 2.2, solvent 1) which crystallised from ethanol-ethyl acetate and, after recrystallisation from the same solvent, had $[\alpha]_D^{16}$ + 85.2°(c 0.74), m.p. and mixed m.p. 147-149° with authentic 2-O-methyl-D-galactose.[110]

Tube 42 contained no carbohydrate.

Tubes 43-54 afforded a chromatographically pure syrup (99 mg; Rgal 1.80, solvent 1) which crystallised from ethanol and, after recrystallisation from ethanol-ethyl acetate, had $[\alpha]_D^{17}$ + 49° (5 mins) → + 73.0° (c 0.63), m.p. and mixed m.p. 202-204° with authentic 4-O-methyl-D-galactose.[110]

Tubes 57-63 contained galactose (28 mg), $[\alpha]_D^{16}$ + 81° (c 0.66), m.p. and mixed m.p. 166-167° with authentic D-galactose. The derived mucic acid had m.p. and mixed m.p. 212-213° with an authentic sample.

2.2.8. Partial hydrolysis of polysaccharide.

(a)- In order to determine the optimum conditions for the production of low molecular weight oligosaccharides, polysaccharide samples (1g) were hydrolysed with 0.5N, 0.75N, and N sulphuric acid on a boiling water bath. Aliquots (1 ml) were withdrawn at regular intervals, neutralised ($BaCO_3$), and analysed by paper chromatography (solvent 1, spray a). Hydrolysis with N acid for 1 h proved to be satisfactory for the production of some oligosaccharides, while others were produced in greater concentration when the hydrolysis was carried out with 0.75 N acid for 0.75 h.

(b)- Polysaccharide (20g; $[\alpha]_D^{16}$ + 63.3°; SO_4^{2-}, 19.62%) was heated on a boiling water bath with 0.75 N sulphuric acid (130 ml) for 0.75 h. Ethanol (240 ml) was added, and the precipitated polysaccharide (0.2g) was removed by filtration. The filtrate was concentrated (150 ml) under reduced pressure, neutralised with barium carbonate, deionised, and evaporated to a mobile syrup (2.8g). In a second hydrolysis experiment polysaccharide (20g) was heated with N sulphuric acid for 1 h, and the mixture of neutral sugars (3.8g) isolated as above. The syrups were combined, dissolved in the minimum quantity of water, and applied to a charcoal-Celite column (1:1; 5.4 x 60 cm). Monosaccharides were eluted with water and oligosaccharides with aqueous alcohol of increasing strength as indicated below. Fractions (ca. 50 ml) were collected and examined by paper chromatography. Like fractions were combined, evaporated to dryness, and weighed. The following products were subsequently identified:

Fraction I (600 mg) was eluted with water (0.5 l). Paper chromatography (solvents 1 and 4) revealed the presence of galactose (major sugar) and xylose. Separation of this

mixture on/.....

mixture on Whatman 3MM (solvent 1), followed by extraction of the appropriate portions of the papers, yielded D-galactose (300 mg), m.p. and mixed m.p. 165-167°, $[\alpha]_D^{16}$ + 76.9° (c 1.56) and D-xylose (15 mg), m.p. and mixed m.p. 145-146°,[81] $[\alpha]_D^{16}$ + 19.0° (c 0.55).

Fraction II (350 mg) was eluted with water (0.4 l). Paper chromatography (solvents 1,3, and 4) revealed spots with the mobilities of galactose, 4-O-methylgalactose, and 2-O-methylgalactose.

Fraction III (260 mg) was eluted with water (0.5 l) and contained 2-O-methylgalactose (major sugar) and 4-O-methylgalactose. Pure 2-O-methylgalactose, m.p. 148-150°, was obtained when this fraction was mixed with ethanol-ethyl acetate.

Fraction IV (244 mg) was eluted with water (1 l) and shown by paper chromatography (solvents 1,2,3, and 4) to be a mixture of 2-O-methylgalactose and 6-O-methylgalactose. The 2-O-methylgalactose readily crystallised from ethanol-ethyl acetate and had m.p. 148-150°. The mother liquor was concentrated to a syrup which was separated on Whatman 3MM (solvent 2), and the portions of the papers corresponding to 6-O-methylgalactose were extracted with water, evaporated to dryness, and the residue crystallised from ethanol. After recrystallisation from the same solvent this sugar had $[\alpha]_D^{16}$ + 76.5°, m.p. and mixed m.p. 118-120° with authentic 6-O-methyl-D-galactose. Nunn and von Holdt[22] reported m.p. 122-123°.

Fraction V (90 mg) was eluted with 4-10% aqueous ethanol (3.0 l) and was shown by paper chromatography (solvent 1) to be a mixture of three oligosaccharides, Rgal 0.21, 0.3, and 0.45.

Fraction VI/.....

Fraction VI (50 mg) was eluted with 4-10% aqueous ethanol (1.5 l) and was shown by paper chromatography (solvent 1) to be a mixture of two oligosaccharides, Rgal 0.21 and 0.3. Repeated separation of this fraction on Whatman No.1 paper (solvent 1) gave a chromatographically pure syrup (Rgal 0.3), which crystallised (4 mg) from aqueous methanol and had m.p. 236-238°, $[\alpha]_D^{16}$ - 51.9° (c 0.24). Paper chromatography (solvent 1) of a partial acid hydrolysate revealed the presence of galactose and the original material. It gave a red colour with triphenyltetrazolium chloride spray reagent and a blue colour with spray c. The infrared spectrum given by this sugar was different from that given by 4-O-β-D-galactopyranosyl-D-galactose.

Fraction VII (295 mg) was eluted with 10% aqueous ethanol (1.7 l). Paper chromatography of the colourless syrup revealed the presence of two sugars, Rgal 0.33 and 0.41 (trace). The main component was readily crystallised from aqueous ethanol and, after recrystallisation from the same solvent, had $[\alpha]_D^{16}$ + 65° (c 0.6), m.p. and mixed m.p. 206-208° with 4-O-β-D-galactopyranosyl-D-galactose. It gave galactose on hydrolysis, readily showed up as a red spot with triphenyltetrazolium chloride spray, and gave a grey-blue colour with spray c. A portion (10 mg) was dissolved in dimethylformamide (2 ml) and methylated with methyl iodide (2 ml) and dry silver oxide (2 g). T.l.c. of the product indicated (spray d) that methylation was incomplete. Hence the product was given three treatments with Purdie's reagents,[113] after which, methylation was found to be complete (t.l.c.). The methylated product was hydrolysed (N sulphuric acid) and examined by t.l.c. (spray c). Spots with the mobilities of 2,3,4,6-tetra-O-methylgalactose (blue-green; RTMG 1) and 2,3,6-tri-O-methylgalactose (grey; RTMG 0.86) were observed. This

oligosaccharide is/.....

oligosaccharide is therefore 4-O-β-D-galactopyranosyl-D-galactose.

Fraction VIII (155 mg) was eluted with 10-15% aqueous alcohol (1 l) and was shown by paper chromatography (solvent 1) to consist mainly of two sugars, Rgal 0.33 and 0.78, together with traces of two other sugars, Rgal 0.41 and 0.49.

Fraction IX (650 mg) was eluted with 10-15% aqueous ethanol (6.7 l). Paper chromatography (solvent 1) revealed the presence of a sugar at Rgal 0.78 and minute traces of three other sugars, Rgal 0.18, 0.33, and 0.49. This fraction readily crystallised when triturated with methanol and, after recrystallisation from the same solvent, had $[\alpha]_D^{16}$ + 87.6° (4 min) → + 70.1° (c 0.69), m.p. 213-214°. It gave a yellow-green colour with spray c and failed to react with triphenyltetrazolium chloride spray. Partial hydrolysis of this sugar, followed by paper chromatography, revealed the presence of galactose, 2-O-methylgalactose, and the original material, while complete hydrolysis revealed the presence of galactose and 2-O-methylgalactose in approximately equal amounts. The sugar (5 mg) was dissolved in water (2 ml), and sodium borohydride (5 mg) added and the mixture allowed to stand for 2 h. The solution was then treated with Amberlite IR-120 [H^+] resin, evaporated, and distilled with methanol to remove borate. Hydrolysis of the non-reducing syrup, followed by paper chromatography (solvent 1), revealed the presence of galactose. A portion (10 mg) was dissolved in dimethylformamide (2 ml) and treated with methyl iodide (2 ml) and dry silver oxide (2 g) to give a partially methylated product which, after three treatments with Purdie's reagents,[113] was found to be completely methylated (t.l.c. spray d). The methylated product was refluxed with 2% methanolic hydrogen

chloride for/.....

chloride for 12 h and the derived methylglycosides examined by g.l.c. Peaks corresponding to 2,3,4,6-tetra-O-methyl-galactose (T 2.00) and 2,3,6-tri-O-methylgalactose (T 3.86, 4.72, 5.35, and 5.90) in the molar ratio 1.5:1.62 were observed. This oligosaccharide is probably 4-O-β-D-galactopyranosyl-2-O-methyl-D-galactose. The β configuration is assumed from the specific rotation of the compound.

Fraction X (260 mg) was eluted with 15% aqueous ethanol (7 l). Paper chromatography (solvent 1) revealed the presence of at least four oligosaccharides, Rgal 0.14, 0.22, 0.78, and 1. This fraction was not further investigated.

Fraction XI (240 mg) was eluted with 15-50% aqueous ethanol (2 l) and was shown by paper chromatography (solvent 1) to consist of a fast moving oligosaccharide (Rgal 1.86) and at least three other oligosaccharides (Rgal 0.1 - 0.22). Separation of this fraction on Whatman No. 1 paper (solvent 1), followed by extraction of the appropriate portions of the papers with methanol and concentration, afforded a chromatographically pure syrup (35 mg). It had $[\alpha]_D^{16}$ + 55.5° (c 0.69), Rgal 1.86 (solvent 1), 1.67 (solvent 2), and 1.55 (solvent 3), and gave a yellow-green colour with spray c, but failed to react with triphenyltetrazolium chloride spray. Partial acid hydrolysis, followed by paper chromatography (solvents 2 and 3), revealed the presence of 2-O-methylgalactose, 6-O-methylgalactose, and starting material. Reduction of the oligosaccharide, followed by paper chromatography of the hydrolysate of the non-reducing syrup, revealed the presence of 6-O-methylgalactose and a minute trace of galactose. A portion of the oligosaccharide (10 mg) was methylated, methanolysed, and the derived methylglycosides examined by g.l.c. Peaks corresponding to 2,3,4,6-tetra-O-methylgalactose

(T 1.98),/.....

(T 1.98), 2,3,6-tri-O-methylgalactose (T 3.85, 4.38, 5.33, and 5.82), and 2,4,6-tri-O-methylgalactose (T 5.33 and 5.82) in the approximate molar ratio of 1.31:0.76:0.60. In addition, a small peak due to 2,3,4-tri-O-methylgalactose (T 9.30) was observed. This sugar is considered to have arisen from demethylation of some of the 2,3,4,6-tetra-O-methylgalactose during methanolysis. The above evidence suggests that this "oligosaccharide" is a mixture of 6-O-methyl-4-O-D-galactopyranosyl-2-O-methyl-D-galactose and 6-O-methyl-3-O-D-galactopyranosyl-2-O-methyl-D-galactose. However, paper chromatography of the oligosaccharide (solvents 1,2,3, and 4) always revealed a single discrete spot.

2.2.9. Quantitative estimation of galactose in the polysaccharide.

This was determined as described in 2.1.4. for aeodan. The percentage of galactose (calc. as $C_6H_{10}O_5$) present in each of the three polysaccharides is recorded in Table 1.

2.2.10. Examination of the polysaccharide in the ultracentrifuge.

The polysaccharide ($[\alpha]_D^{16}$ + 81.1°; SO_4^{2-}, 19.48%) was examined at a concentration of 0.5% in 0.1M sodium phosphate buffer at an ultracentrifuge speed of 56,000 r.p.m.

3. DISCUSSION

3.1. Aeodes orbitosa

Aeodes orbitosa, a red seaweed belonging to the Grateloupiaceae is easily recognised by its broad flat fronds. The seaweed grows abundantly on the Atlantic coast of the Cape Peninsula. In some areas the seaweed attains an enormous size and resembles a cloak. The weed used in the present investigation was collected at Sea Point, near Cape Town.

Hot water extraction of the fresh weed, followed by centrifugation and precipitation into ethanol of the aqueous extract, yielded a highly sulphated polysacchardie (9.5% on a wet wt. basis), aeodan, which was contaminated with nitrogenous material. The residual weed contained an insignificant amount of polysaccharide and was not further investigated. The crude polysaccharide was purified by dissolution in water, centrifugation of the solution, followed by precipitation into ethanol. After several such cycles, the polysaccharide was converted to the free acid form (Amberlite IR-120[H^+] resin) which was neutralised with sodium hydroxide solution, and the polymer recovered by precipitation into ethanol.

Aeodan was examined in the ultracentrifuge in phosphate buffer, and only a single sharp peak was observed, even after 164 min of centrifugation at 56, 100 r.p.m. Very little diffusion of the sedimentation peak occurred during the run; this tends to confirm the homogeneous nature of the polysaccharide, and is indicative of an extended type of molecule.

Aeodan had a specific rotation of + 79°, contained 27.5% of sulphate (calc. as $NaSO_3^-$), and gave a negative test for 3,6-anhydrogalactose (found 0.1%).[97] It failed to precipitate from aqueous solution when mixed with potassium chloride solution, and hence does not appear to be a κ-carrageenan

type polysaccharide/.....

type polysaccharide. Chromatography of a hydrolysate of aeodan revealed the presence of galactose (major sugar), 2-O-methylgalactose, and 6-O-methylgalactose together with trace amounts of xylose, glycerol, and 4-O-methylgalactose. This mixture was separated on a cellulose column to yield pure glycerol, D-galactose, and 2-O-methyl-D-galactose. Separation of mixed fractions by chromatography on thick paper resulted in the isolation of D-xylose, 4-O-methyl-L-galactose, and 6-O-methyl-D-galactose. All the sugars were obtained in crystalline form and were characterised by their optical rotations, melting points, and mixed melting points with authentic samples where available. In addition, galactose and 2-O-methyl-D-galactose were further characterised by conversion to mucic acid and 2-O-methyl-N-phenyl-D-galactosylamine respectively, while glycerol was characterised as the tri-p-nitrobenzoate. As far as the author is aware this is the first recorded instance of 2-O-methylgalactose in Nature. In demonstrating the presence of glycerol as a polysaccharide component, special care (see Experimental) was taken to ensure that it had not arisen as a contaminant from an exterior source.

The yield of galactose (58%, calc. as $C_6H_{10}O_5$) was determined by the Somogyi micromethod,[102] after hydrolysis and separation by paper chromatography. The content of mono-O-methylgalactoses (10.8%, calc. as $C_7H_{12}O_5$) was calculated from the methoxyl value (1.9%) of the polysaccharide. Attempts to estimate the glycerol have so far been unsuccessful. However, on the amount isolated from large scale hydrolysis of the polysaccharide, it is estimated to be present to the extent of 1-2%. From the above values, the approximate molecular ratio of D-galactose : mono-O-methylgalactoses: sulphate ($NaSO_3^-$) is 12:2:9. Such a repeating unit has an equivalent weight of 366 (found 375) and a methoxyl content of 1.88%. No account has been taken of glycerol in these

calculations./.....

calculations.

It has been established from experiments on monosaccharide and polysaccharide sulphates[66,73] that galactose units carrying ester sulphate at position-6 and having position-3 free or vice versa undergo elimination of sulphate with concomitant 3,6-anhydride formation when treated with alkali. In addition, sulphate ester groups which are adjacent and *trans* to a free hydroxyl group are labile to alkali.[81,88] Treatment of such sulphate groups with sodium methoxide causes their cleavage and the intermediate formation of epoxide rings. Attack by the methoxide ion on either side and *trans* to the epoxide oxygen can then occur with the formation of monomethyl sugars. In the present experiments the action of alkali on aeodan in the presence of borohydride[66] to prevent end-group degradation, resulted in the liberation of *ca.* 16% of the sulphate and the formation of 5.1% of 3,6-anhydrosugar.[97] This indicates that *ca.* 5.7% of the hexose residues are substituted in either the 2- or 4-positions or partly in each. This substitution could be interpreted as 1,2- or 1,4-linked galactose-6 (or -3)-sulphate and/or 1,4-linked 2-*O*-methyl-D-galactose-6 (or -3)-sulphate and/or 1,2-linked 4-*O*-methyl-L-galactose-6 (or -3)-sulphate.

It has been observed that there is not an exact molar correspondence between the amount of sulphate released and 3,6-anhydride formed, but an excess of released sulphate amounting to *ca.* 13% of the total eliminated. This disparity could be due to the presence of other alkali-labile sulphate groups in the polysaccharide, or the occurrence of a certain amount of O-S cleavage of alkali-stable groups under the alkaline conditions. To distinguish between these two possibilities aeodan was refluxed with sodium methoxide for 24 h, and the derived polysaccharide hydrolysed and examined chromatographically. No monomethyl pentose,

dimethyl hexose/.....

dimethyl hexose or new monomethyl hexose was observed, thus suggesting the absence of additional alkali-labile sulphate groups.

Infrared spectra of carbohydrate sulphates[111] show a general absorption band at 1240 cm^{-1}, and, in addition, bands that are thought to be specific for the type of sulphate ester present; thus galactose-6-sulphate shows a peak at 820 cm^{-1} (sulphated primary hydroxyl group), galactose-2-sulphate (the esterified hydroxyl group being equatorially disposed in the stable C1 conformation) shows a peak at 830 cm^{-1}, and galactose-4-sulphate (the esterified hydroxyl group being axially disposed in the stable C1 conformation) shows a peak at 850 cm^{-1}. The infrared spectrum of aeodan (Figure 1) showed a peak at 1240 cm^{-1} and one at 815 cm^{-1} suggestive of the presence of sulphated primary hydroxyl. This peak was very broad, the peak area spreading well past the 830 cm^{-1} wave length, and possibly masking a second peak in this area, which would be indicative of equatorial secondary sulphate (possibly at position-2).

Periodate[81] and methylation[49] studies on polysaccharides, before and after removal of the ester sulphate groups, have provided information about the site of the sulphate groups. The preparation of a sample of desulphated aeodan (with the minimum amount of degradation) was therefore necessary in order that aeodan and desulphated aeodan could be studied by these techniques. No conditions were found for the complete desulphation of the molecule; the lowest sulphate values were achieved by shaking with 0.15M methanolic hydrogen chloride[85] for 114 h at room temperature. This treatment gave a yield of 36% of degraded polysaccharide having 3.45% sulphate. Paper chromatography of an acid hydrolysate of the methanol soluble material revealed that desulphation had removed some of the galactose, 2-O-methyl-,

and 6-O-methylgalactose/.....

Infrared spectrum of aeodan

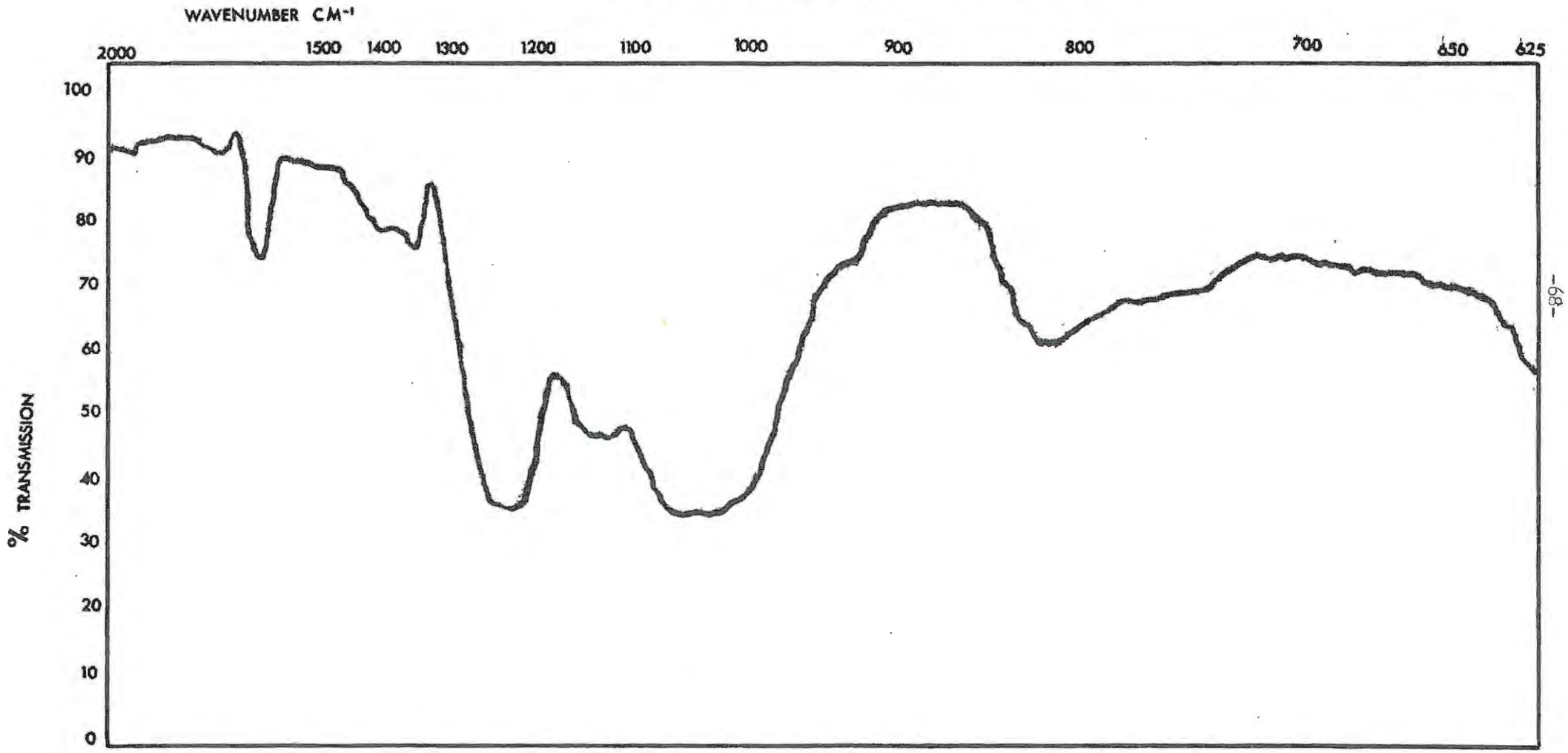


Figure 1

and 6-O-methylgalactose units in addition to trace amounts of xylose and 4-O-methylgalactose. The infrared spectrum of the desulphated polysaccharide (Figure 2) displayed a peak at 1240 cm^{-1}, but no other well defined peaks in the 800-860 cm^{-1} region.

Oxidation of aeodan with periodate ceased after 48 h at room temperature (Table 2a and 2b), when 0.170 (0.213) mole of periodate had been consumed per C_6 anhydro unit (per sulphate free anhydrohexose unit). In the case of each specimen of desulphated aeodan no definite end point was reached, even after 120 h. However, the rate of reduction was then extremely low. After this time both specimens had consumed about four (three and a half) times as much periodate per C_6 anhydro unit (sulphate free anhydrohexose unit) as aeodan.

Table 2a.

MOLE PERIODATE REDUCED PER C_6 ANHYDRO UNIT

Time (h)	3	24	48	72	96	120
Aeodan	0.051	0.142	0.170	0.170	0.170	
Desulphated aeodan (SO_4^{2-}, 6.3%)	0.456	0.610	0.675	0.698	0.718	0.723
Desulphated aeodan (SO_4^{2-}, 3.45%)	0.444	0.545	0.607	0.634	0.641	0.659

The accurate determination of the true reduction of periodate for every sugar residue in a sulphated heteropolysaccharide is complex, and the reduction of periodate in terms of "C_6 anhydro unit" is usually chosen for simplicity and is calculated on the assumption that the polysaccharide is a homohexan. Such results are of value for comparison with those of oxidation studies on substances of similar composition. However, results of periodate oxidation studies

on sulphated/.....

Infrared spectrum of desulphated aeodan

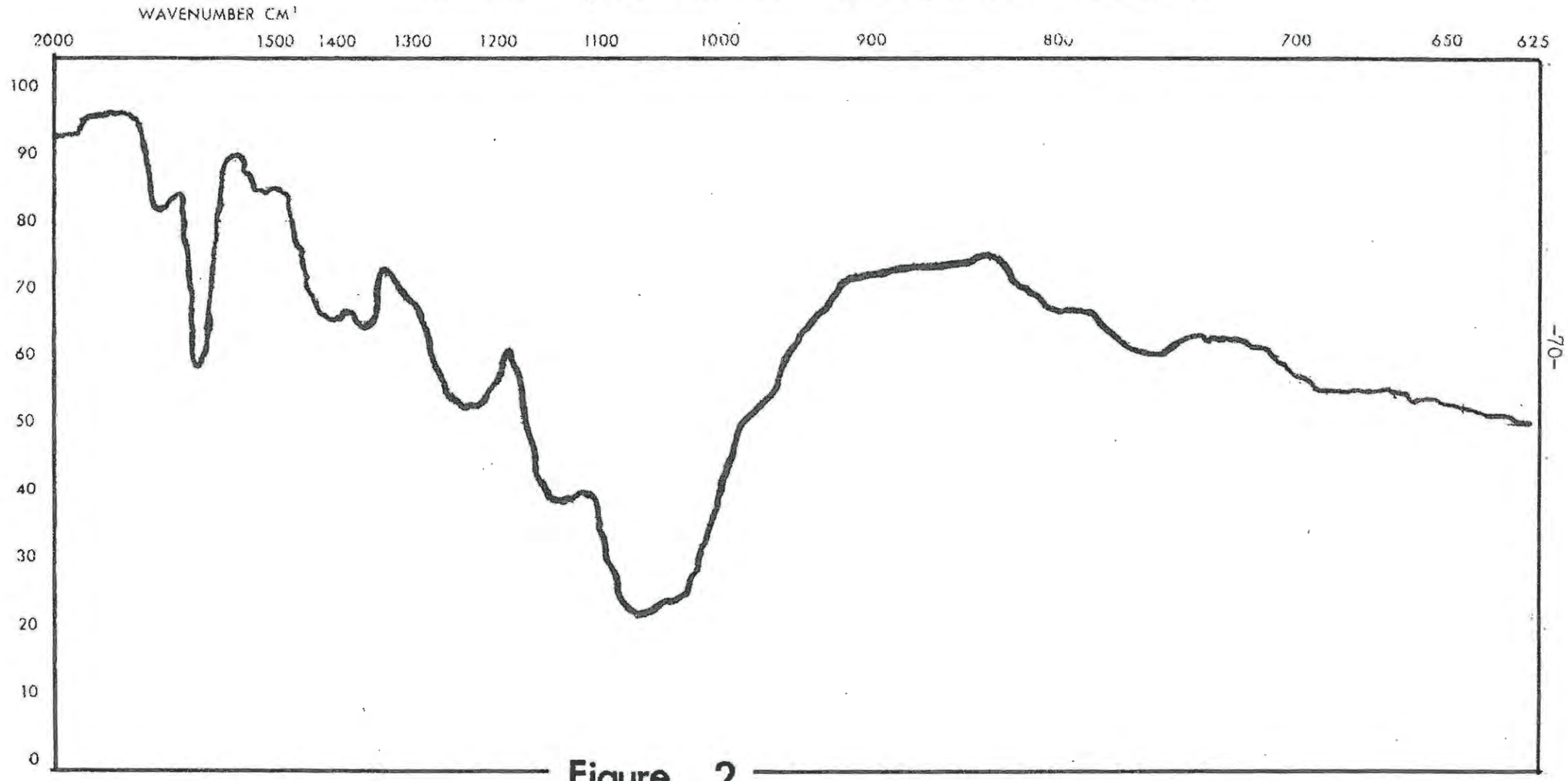


Figure 2

on sulphated polysaccharides expressed in this manner cannot be used for direct comparison with those of oxidation studies on similar desulphated polysaccharides. If, on the other hand, allowance is made for the different sulphate content of these polysaccharides and the results of periodate reduction are expressed on a sulphate free basis *viz.* per sulphate free anhydrohexose unit, then direct comparisons become more valid (see Table 2b)

Table 2b

MOLE PERIODATE REDUCED PER SULPHATE FREE ANHYDROHEXOSE UNIT

Time (h)	3	24	48	72	96	120
Aeodan	0.070	0.196	0.213	0.213	0.213	
Desulphated aeodan (SO_4^{2-}, 6.3%)	0.490	0.654	0.724	0.742	0.770	0.776
Desulphated aeodan (SO_4^{2-}, 3.45%)	0.461	0.566	0.630	0.660	0.667	0.684

The low consumption of periodate by aeodan suggests either the presence of a large proportion of 1,3 links; or the presence of 1,3 links together with units containing other glycosidic links, but carrying sulphate or methoxyl groups in such positions as to render these units immune to periodate; or the presence of a large proportion of units containing other glycosidic links carrying sulphate or methoxyl groups in such positions as to render these units immune to periodate.

Aeodan and desulphated aeodan were oxidised with periodate, and the resulting oxopolysaccharides reduced with borohydride.[104] Complete acid hydrolysis of the polyalcohols, followed by paper chromatography, revealed the presence of threitol, glycol aldehyde, and a trace of glycerol as degradation products of aeodan together with unchanged galactose, 2-*O*-methylgalactose,

6-O-methylgalactose, and 4-O-methylgalactose, whereas only threitol, glycol aldehyde, and the above four sugars were detected in the case of the polyalcohol from desulphated aeodan. No xylose was detected on any of the chromatograms indicating that these units are either 1,2- or 1,4-linked in the polymer. Most of the threitol and glycol aldehyde are considered to have arisen from galactose units substituted at position-4, probably by glycosidic links, and unsubstituted in positions-2, -3, and -6; evidence which is supported by the presence of 2,3,6-tri-O-methyl-D-galactose in the hydrolysis products of methylated aeodan and methylated desulphated aeodan. In addition, alkali treatment of aeodan indicates that only a small portion of the galactose units carry sulphate on positions-6 (or -3). The presence of glycerol in the degradation products of aeodan could have arisen (1) from glycerol glycosidically linked through position-2 as an end group, or (11) through oxidation of 6-substituted galactose units having positions-2, -3, and -4 free, or (111) through oxidation of 4-linked xylose units having positions-2 and -3 free. The second explanation is partly supported by the presence of a trace of 2,3,4-tri-O-methylgalactose in the hydrolysate of methylated aeodan. However, not only could the trace of tri-O-methylgalactose have arisen from undermethylation, but no glycerol was detected in the hydrolysate of the polyalcohol obtained from desulphated aeodan, results which tend to rule out this explanation for the presence of glycerol in the hydrolysate. The third explanation is supported by the absence of xylose from the hydrolysate of the polyalcohol derived from aeodan. However, the absence of glycerol from the hydrolysate of the polyalcohol derived from desulphated aeodan tends to rule out this explanation, although it is possible that glycerol was present in the hydrolysate, but at so low a concentration, since xylose units were cleaved during

the desulphation/.....

the desulphation of aeodan with methanolic hydrogen chloride, that it could not be detected chromatographically. The only other possibility, viz. 2-substituted galactose having positions-3, -4, and -6 free, is ruled out by the absence of glyceraldehyde in the hydrolysate of the polyalcohol obtained from aeodan.

The presence of 6-O-methylgalactose residues in the polyalcohols derived from aeodan and desulphated (6.3% of sulphate) aeodan indicates that these units are 1,3-linked. If these units were glycosidically linked through position-4 and carried sulphate (it is possible that the small amount of sulphate present in desulphated aeodan is largely attached to the 6-O-methylgalactose units) at position-3 or -2, then they would be immune to periodate. However, such units would be labile to sodium methoxide with di-O-methylhexose formation, and are therefore ruled out. The alternative units viz. 1,4-linked 2,3-disulphate, 1,2-linked 3- or 4-sulphate, and 1,2-linked 3,4-disulphate are all ruled out by the absence of 6-O-methylgalactose in the hydrolysate of methylated aeodan and the absence of 3,4,6-tri-O-methylgalactose in the hydrolysis products of methylated desulphated aeodan.

Methylation of desulphated aeodan by a modification of Kuhn and Trischmann's method,[112] followed by several treatments with Purdie's reagents,[113] afforded a product having a methoxyl content of 35%. Infrared examination of this material revealed a very small hydroxyl peak. Further treatment with Purdie's reagents[113] failed to increase the methoxyl content. It was therefore concluded that the low methoxyl content found for methylated desulphated aeodan was due to contamination. Hydrolysis of the methylated polysaccharide, followed by separation of the products by paper chromatography, afforded 2,3,4,6-tetra-O-methylgalactose, a tri-O-methylgalactose fraction, and a di-O-methyl fraction. Separation of the tri-O-methyl fraction from a charcoal-Celite

column by/.....

column by gradient elution with aqueous methyl ethyl ketone gave pure 2,4,6-tri- and 2,3,6-tri-O-methyl-D-galactose. The di-O-methyl fraction was shown by paper chromatography to consist of approximately equal amounts of 2,6- and 4,6-di-O-methylgalactose. This fraction is considered to have arisen from the sulphated hexose residues present in desulphated aeodan. The major products of the hydrolysis *viz.* 2,4,6- and 2,3,6-tri-O-methylgalactose confirms the presence of 1,3- and 1,4-linkages in aeodan. In addition, the high yield of these two sugars (*ca.* 80% of the methylated units isolated) strongly suggests that aeodan is composed entirely of 1,3- and 1,4-linkages.

In order to obtain evidence for the location of the sulphate ester groups, it was decided to methylate aeodan with the sulphate groups intact. In common with other highly sulphated polysaccharides, aeodan was difficult to methylate and a maximum methoxyl content of 19.8% only was achieved. The final product contained 16.5% sulphate and gave a faint positive reaction for 3,6-anhydrogalactose.[97] The methylated polysaccharide was hydrolysed with N sulphuric acid, and the products were separated on a cellulose column with half-saturated butanol. 2,3,4,6-Tetra-O-methyl-D-galactose, a mixture of 2,4,6- and 2,3,6-tri-O-methyl-D-galactose containing a trace of 2,3,4-tri-O-methylgalactose, a mixture of 4,6- and 2,6-di-O-methyl-D-galactose, 4,6-di-O-methyl-D-galactose, a mixture of 4,6-di- and 4-O-methyl-D-galactose, and 4-O-methyl-D-galactose were obtained. Separation of the tri-O-methylgalactose fraction from a charcoal-Celite column by gradient elution with aqueous methyl ethyl ketone gave pure 2,4,6- and 2,3,6-tri-O-methyl-D-galactose, while separation of the di-O-methyl fraction from a cellulose column with ethyl acetate-acetic acid-formic acid-water (18:3:1:4) afforded crystalline 2,6- and

4,6-di-O-methyl-D-galactoses. The methylated sugars were characterised as crystalline materials and where necessary by conversion to crystalline derivatives.

The results of the methylation of aeodan must be treated with some caution because of the uncertainty of the extent of methylation. The interpretation of the isolation of 4,6-di- and 2,4,6-tri-O-methyl-D-galactose is quite unambiguous; both of these units are derived from the 1,3-linked units that were shown to be present by methylation analysis of desulphated aeodan. The presence of 1,3-linked galactose-2-sulphate units which is clearly shown by the isolation of the 4,6-di-O-methyl-D-galactose, was suspected from the infrared spectrum of aeodan and the stability of most of the sulphate ester groups in aeodan to alkali. The 2,6-di-O-methyl-D-galactose could have arisen from 1,3-linked galactose-4-sulphate, although the spectroscopic evidence (no band at 850-860 cm^{-1} for axial secondary sulphate) is against this. On the other hand, it may represent a genuine 1,4-linked unit or, possibly, undermethylation. The size of the peak at 815 cm^{-1} recorded in the infrared spectrum of aeodan cannot be accounted for by the small quality of sulphate which underwent elimination with concomitant 3,6-anhydride formation when aeodan was treated with alkali. Hence the 4-O-methyl-D-galactose isolated in good yield from the hydrolysate of methylated aeodan must have arisen from 1,3-linked galactose units carrying sulphate at positions-2 and -6. In view of the uncertainty of the extent of methylation it could be argued that the 4-O-methyl-D-galactose units are due to undermethylation. However, the resistant nature of the hydroxyl group at C_4 of galactose units to methylation, especially when such units are glycosidically linked at position-3 rules out this argument. The 2,3,6-tri-O-methyl-D-galactose units must have arisen from 1,4-linked units present in aeodan.

Partial acid/.....

Partial acid hydrolysis of aeodan, followed by separation of the neutral material on Whatman 3MM paper, resulted in the isolation of 3-O-β-D-galactopyranosyl-D-galactose and 4-O-β-D-galactopyranosyl-D-galactose. The former had $[\alpha]_D$ + 60° and gave only galactose on hydrolysis. Methylation of this sugar, followed by hydrolysis and separation of the products on Whatman No 20 paper, resulted in the isolation and characterisation of 2,4,6-tri- and 2,3,4,6-tetra-O-methyl-D-galactose. The optical rotation of this hygroscopic sugar was extremely difficult to determine accurately due to the cloudiness of the solution. All attempts to prepare an optically clear solution failed. Hence, although the β-configuration has been assigned to this sugar it should not be considered definite. The crystalline disaccharide had a melting point of 205-208° and a specific rotation of + 73.2°. It gave only galactose on hydrolysis. Gas chromatography of the methanolysed methylated material revealed the presence of methyl 2,3,6-tri- and 2,3,4,6-tetra-O-methylgalactoside. The isolation of these two disaccharides confirms the presence of 1,4- and 1,3-links in aeodan.

At present, no unique structure can be proposed for aeodan. It can be seen that the experimental results are complementary to one another and that aeodan contains the following structural units: β 1,4-linked galactose, 1,3-linked galactose, 1,3-linked galactose-2-sulphate, 1,3-linked galactose-2,6-disulphate, and 1,3-linked 6-O-methyl-D-galactose. Aeodan resembles the λ-fraction of carrageenan in several respects. It is not precipitated from solution in the presence of potassium chloride, it is highly sulphated, and it contains 1,3-linked galactose-2-sulphate units. On the other hand, aeodan differs from λ-carrageenan in having most of the 1,4-linked galactose units free of sulphate and therefore a lower proportion of alkali labile sulphate.

In addition/.....

In addition, the 2,6-disulphate units which are 1,3-linked in aeodan are 1,4-linked in λ-carrageenan.

3.2. Phyllymenia cornea.

Phyllymenia cornea, a red seaweed belonging to the Grateloupiaceae, is fairly easily recognised by its broad, bright-red fronds. The weed, found in fair quantity in certain areas along the east coast of Southern Africa, grows mainly in deep water and is best collected from the drift after spring tide.

Exhaustive hot water extraction of fresh wet Phyllymenia cornea, followed by centrifugation and precipitation into ethanol of the mucilage, afforded a sulphated polysaccharide, phyllymenan. Purification of phyllymenan was effected by dissolution in water, centrifugation, and precipitation into ethanol. Phyllymenan was extracted and purified from three different batches of seaweed (see Table 1). The weed was found growing at Kowie Point, (ca. 120 miles from Port Elizabeth), but not at Palmiet (ca. 130 miles from Port Elizabeth) where it was collected from the drift. All three samples of phyllymenan failed to precipitate from aqueous solution when mixed with potassium chloride solution, and hence phyllymenan does not appear to be a κ-carrageenan type polysaccharide. Two of the three samples of phyllymenan had identical specific rotations. The lower specific rotation of the third sample can most probably be attributed to contamination of the polysaccharide with protein (found 0.48% N). All three samples of phyllymenan contained similar amounts of ester sulphate, and like acodan contained galactose, 2-O-methyl-D-galactose, and 6-O-methyl-D-galactose together with smaller amounts of xylose and 4-O-methyl-L-galactose.

The quantity of galactose (calc. as $C_6H_{10}O_5$) present in each sample of phyllymenan (see Table 1) was determined by the Somogyi micromethod,[102] after hydrolysis, and separation by paper chromatography. The quantity of mono-O-methylgalactoses (calc. as $C_7H_{12}O_5$)/.....

toses (calc. as $C_7H_{12}O_5$) present in each sample of phyllymenan, calculated from the methoxyl values, was found to vary from 24 to 29%. In this respect phyllymenan differs from aeodan which contained only *ca.* 11% of mono-*O*-methylgalactoses. The predominant mono-*O*-methylgalactose present in aeodan was found to be 6-*O*-methyl-D-galactose, whereas 2-*O*-methyl-D-galactose was found to be the predominant mono-*O*-methylgalactose in each of the samples of phyllymenan. The ratio of hexose to sulphate in the three samples of phyllymenan was approximately 42:20, whereas aeodan contained these residues in the approximate ratio of 42:27. All three samples of phyllymenan contained *ca.* 0.5% of 3,6-anhydrogalactose.[97]

The products of hydrolysis of a sample of phyllymenan were separated on a cellulose column using half saturated butanol. Galactose, 2-*O*-methyl-D-galactose, and 4-*O*-methyl-L-galactose were obtained as crystalline sugars and were characterised by their optical rotations, melting points, and mixed melting points with authentic samples. Galactose was further characterised by conversion to mucic acid. In addition, xylose and 6-*O*-methylgalactose were chromatographically detected in the mixed fractions, but it was not possible to separate pure samples of these sugars by paper chromatography. However, a fraction containing galactose and xylose and one containing 2-*O*-methylgalactose and 6-*O*-methylgalactose was obtained when the partial hydrolysate of phyllymenan was separated from a charcoal-Celite column and it was possible to separate pure samples of xylose and 6-*O*-methylgalactose from these two fractions (see later). No glycerol was detected in the hydrolysis products of any of the samples of phyllymenan.

A sample of phyllymenan was examined in the ultracentrifuge in phosphate buffer, and only a single peak was observed even after 188 min of centrifugation. The peak given by

phyllymenan was/.....

phyllymenan was not as sharp as that given by aeodan when examined under similar conditions. Nevertheless the sample appeared to be homogeneous. The infrared spectrum of phyllymenan (Figure 3) exhibited the general absorption band for ester sulphate at 1240 cm^{-1}, but did not show any well defined bands for axial, equatorial or primary ester sulphate (800-860 cm^{-1}).[111]

Treatment of phyllymenan with alkali in the presence of borohydride[66] to minimize end group degradation resulted in an 89% yield of polysaccharide containing 17.67% sulphate. Although a reduction in sulphate content of 1.6% occurred during this treatment, estimation of the 3,6-anhydrogalactose[97] content of the alkali modified polysaccharide revealed that no 3,6-anhydrogalactose was formed. Hence, phyllymenan differs from aeodan in not containing any 1,2- or 1,4-linked galactose-6 (or -3)-sulphate units. Hydrolysis of alkali modified phyllymenan, followed by paper chromatography, revealed the presence of galactose, 2-O-methylgalactose, 6-O-methylgalactose (trace), 4-O-methylgalactose (trace), and xylose (trace). The small reduction in sulphate content which occurred during alkali treatment of phyllymenan was most probably the result of O-S cleavage of alkali stable groups.

Desulphation of aeodan with methanolic hydrogen chloride[85] was accompanied by fairly extensive degradation of the molecule, and hence no information could be obtained concerning the positions of the sulphate ester groups in aeodan by a comparison of the quantity of periodate reduced by aeodan and desulphated aeodan. In addition, no conditions were found for the complete desulphation of aeodan. In the case of phyllymenan complete desulphation of the polysaccharide was achieved by shaking the polysaccharide at room temperature for 48 h with 0.15M methanolic hydrogen chloride.[85] This treatment gave a 69% yield of desulphated polysaccharide

(found 0.0%/.....

Infrared spectrum of phyllymenan

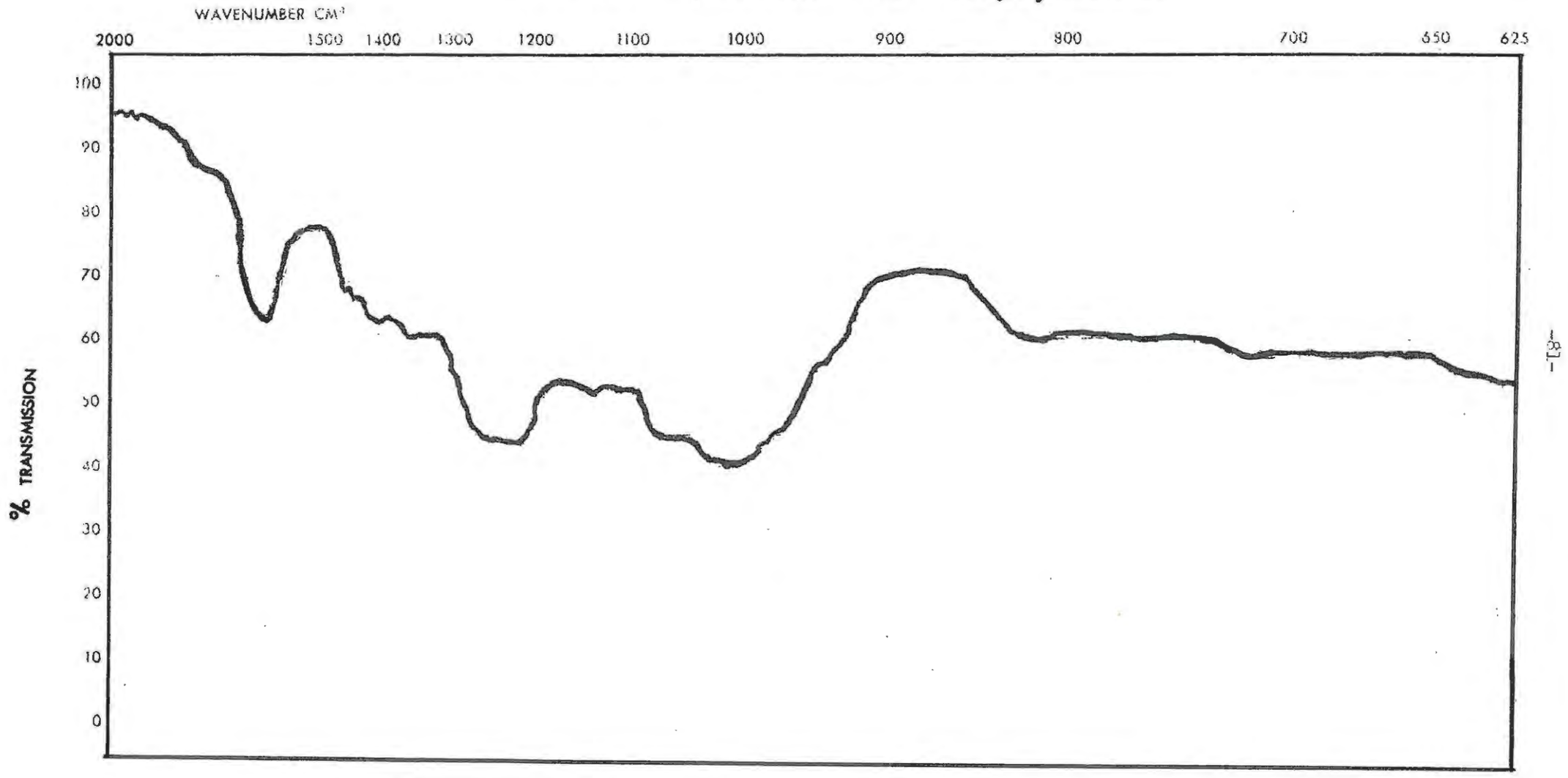


Figure 3

(found 0.0% sulphate). Paper chromatographic examination of a hydrolysate of the non-reducing methanol soluble material revealed that galactose, 6-O-methylgalactose, and traces of 2-O- and 4-O-methylgalactose were cleaved during the desulphation process. Calculation revealed that less than 10% of the sugar units present in phyllymenan were removed during the desulphation process. Treatment of phyllymenan with 0.1M methanolic hydrogen chloride at room temperature for 48 h and 72 h reduced the sulphate content of the polysaccharide from 19.28% to 8% and 4.7% respectively.

It is well known that polysaccharides containing uronosyl linkages are resistant to hydrolysis and undergo very little degradation when desulphated with methanolic hydrogen chloride.[85] In the present investigation no uronic acid was detected in any of the samples of phyllymenan, and hence the stability of the glycosidic linkages to cleavage in phyllymenan can be ascribed to the shielding effect of the large number of methoxyl groups present in the macromolecule.

Oxidation of phyllymenan with periodate ceased after 72 h at room temperature (Table 3a and 3b) when 0.121 (0.153) mole of periodate had been consumed per C_6 anhydro unit (per sulphate free anhydrohexose unit). In the case of desulphated and partially desulphated phyllymenan the reduction of periodate ceased after 24 h at room temperature. After this time desulphated phyllymenan had consumed 0.161 (0.161) mole per C_6 anhydro unit (per sulphate free anhydrohexose unit) while partially desulphated phyllymenan had consumed 0.149 (0.157) mole per C_6 anhydro unit (per sulphate free anhydrohexose unit).

Table 3a/.....

Table 3a

MOLE PERIODATE REDUCED PER C_6 ANHYDRO UNIT

Time(h)	5	24	48	72	96
Phyllymenan	0.067	0.092	0.111	0.121	0.121
Desulphated phyllymenan	0.150	0.161	0.161	0.161	0.161
Partially desulphated phyllymenan (SO_4^{2-}, 4.7%)	0.116	0.149	0.149	0.149	0.149

Table 3b

MOLE PERIODATE REDUCED PER SULPHATE FREE ANHYDROHEXOSE UNIT

Time(h)	5	24	48	72	96
Phyllymenan	0.085	0.116	0.140	0.153	0.153
Desulphated phyllymenan	0.150	0.161	0.161	0.161	0.161
Partially desulphated phyllymenan (SO_4^{2-}, 4.7%)	0.122	0.157	0.157	0.157	0.157

The low consumption of periodate by phyllymenan suggests either the presence of a large proportion of 1,3 links; or the presence of 1,3 links together with units containing other glycosidic links, but carrying sulphate or methoxyl groups in such positions as to render these units immune to periodate; or the presence of a large proportion of units containing other glycosidic links carrying sulphate or methoxyl groups in such positions as to render these units immune to periodate. The reduction of periodate (calculated on a sulphate free basis; Table 3b) by phyllymenan, desulphated phyllymenan, and partially desulphated phyllymenan was approximately the same. This indicates that desulphation of phyllymenan does not result in the production of any new α-glycol groups, which is further

evidence for/.....

evidence for the presence of a large proportion of 1,3 links in the polysaccharide. Phyllymenan was methylated under an atmosphere of nitrogen according to Haworth's method.[114] The product, which was isolated by freeze-drying, was found to be incompletely methylated, even after eighteen additions of dimethyl sulphate and alkali had been made. The partially methylated polymer was therefore dispersed in dry dimethyl sulphoxide and given several treatments with Purdie's reagents.[113] The final product had a methoxyl content of 24.7%. The methylated polymer was hydrolysed with N sulphuric acid and the products were eluted from a cellulose column with ethyl acetate-acetic acid-formic acid-water (18:3:1:4). 2,3,4,6-Tetra-O-methylgalactose, 2,3,6-tri-O-methyl-D-galactose, a mixture of 2,3,6- and 2,4,6-tri-O-methylgalactoses, a mixture containing 2,3,6-tri, 2,4,6-tri, and 2,6-di-O-methyl-D-galactose together with traces of 2,3,4-tri-O-methylgalactose and an unidentified sugar, 2,6-di-O-methyl-D-galactose, a mixture of 2,6- and 4,6-di-O-methylgalactoses, 4,6-di-O-methyl-D-galactose, 2-O-methyl-D-galactose, and galactose were obtained.

The results of the methylation of phyllymenan must be treated with some caution because of the extreme difficulty encountered in methylating the polysaccharide, and hence the uncertainty of the extent of methylation. The 2,3,4,6-tetra-O-methylgalactose, which was not detected in the hydrolysate of partially methylated phyllymenan, is considered to have arisen as a result of degradation of the polymer during the methylation procedure with Purdie's reagent[113] and therefore does not represent the presence of branching in phyllymenan. It is the author's opinion that some 2,3,4,6-tetra-O-methyl-galactose would have been detected in the partially methylated polymer if branching occurred in phyllymenan as the end groups, because of their position, would have become fully methylated after eighteen treatments with dimethyl sulphate and alkali.

The 2,4,6-/.....

The 2,4,6-tri-O-methylgalactose is considered to have arisen from 1,3-linked units, while the isolation of 2,3,6-tri-O-methyl-D-galactose indicates the presence of 1,4 links in phyllymenan. The isolation of 4,6-di-O-methyl-D-galactose suggests the presence of 1,3-linked galactose-2-sulphate units. The alternative units *viz*. 1,2-linked 6-O-methyl-D-galactose-3-sulphate, 1,2-linked galactose-3-sulphate, and 1,2-linked 4-O-methyl-L-galactose-3-sulphate are ruled out because of their instability towards alkali and/or because removal of sulphate from such units results in the production of α-glycol groups. The 2,6-di-O-methyl-D-galactose could have arisen from 1,3-linked units carrying sulphate at position-4 or 1,4-linked 3-sulphate units. The latter is labile to alkali and is therefore ruled out. On the other hand, it may be due to undermethylation. The 4-O-methyl-D-galactose isolated in good yield is considered to have arisen from 1,3-linked galactose units carrying sulphate at positions -2 and -6. The resistant nature of the C_4 hydroxyl group of galactose to methylation especially when glycosidically linked through position-3 tends to rule out the possibility of this sugar arising from undermethylation. The low optical rotation observed for the 4-O-methyl ether suggests the presence of *ca*. 5% of the L isomer. The presence of 2-O-methyl-D-galactose and D-galactose in the hydrolysate of methylated phyllymenan is difficult to explain. These units most probably arose as a result of undermethylation.

Partial acid hydrolysis of phyllymenan, followed by separation of the neutral products from a charcoal-Celite column, yielded four monosaccharide and several oligosaccharide fractions. Since xylose and 6-O-methylgalactose had only been tentatively identified by paper chromatography the opportunity was taken, while isolating the oligosaccharides, of isolating and characterising these two sugars. Both sugars were

obtained as crystalline materials and were characterised as D-xylose and 6-O-methyl-D-galactose by their melting points, mixed melting points with authentic samples, and optical rotations. The major oligosaccharides isolated were 4-O-β-D-galactopyranosyl-D-galactose and 4-O-β-D-galactopyranosyl-2-O-methyl-D-galactose. The former had $[\alpha]_D$ + 65°, melting point and mixed melting point 206-208° with authentic 4-O-β-D-galactopyranosyl-D-galactose. It gave only galactose on hydrolysis. The infrared spectrum given by this sugar was identical with that given by the authentic sugar. Methylation of the sugar, followed by t.l.c. of the hydrolysate, revealed the presence of 2,3,4,6-tetra- and 2,3,6-tri-O-methylgalactose. The latter oligosaccharide had $[\alpha]_D$ + 70.1°, melting point 213-214°, and gave galactose and 2-O-methylgalactose on hydrolysis. Reduction of the oligosaccharide with borohydride, followed by paper chromatography of the hydrolysate of the non-reducing material, revealed that 2-O-methyl-D-galactose occupied the reducing end of the molecule. This was confirmed when the oligosaccharide failed to react with triphenyltetrazolium chloride spray. G.l.c. of the methanolysed methylated oligosaccharide revealed the presence of methyl 2,3,4,6-tetra-O-methylgalactoside and methyl 2,3,6-tri-O-methylgalactoside in the molar ratio 1.5:1.62. In addition, smaller amounts of a galactosylgalactose and a methylated oligosaccharide were isolated from the partial hydrolysate. The former had $[\alpha]_D$ - 51.9°, melting point 236-238°, and gave galactose and unchanged material on hydrolysis. The infrared spectrum given by this sugar was different from that given by 4-O-β-D-galactopyranosyl-D-galactose. It appears that this disaccharide is composed of D- and L-galactose. The possibility of this disaccharide being in the furanose form is ruled out by its resistance to acid hydrolysis. The methylated oligosaccharide was obtained as a syrup and had $[\alpha]_D$ + 55.5°. It gave

2-O-methylgalactose, 6-O-methylgalactose, and unchanged material on partial acid hydrolysis. Reduction of this oligosaccharide with borohydride, followed by paper chromatography of a hydrolysate of the non-reducing material, revealed a major spot with the mobility of 6-O-methylgalactose and a very faint spot with the mobility of galactose. The minute trace of galactose most probably resulted from demethylation of some of the 6-O-methylgalactose during the reduction procedure. G.l.c. examination of the methanolysed methylated oligosaccharide revealed peaks due to methyl 2,3,4,6-tetra-O-methylgalactoside and methyl 2,3,6-tri-O-methylgalactoside. The peaks at T 5.33 and 5.82 were abnormally large especially the one at T 5.33. Since the T values of methyl 2,4,6-tri-O-methylgalactoside coincide with these two T values it was concluded that the abnormality was due to the presence of some methyl 2,4,6-tri-O-methylgalactoside. Calculations (based on the area under the peak at T 3.85 equals 100% methyl 2,3,6-tri-O-methylgalactoside) indicated that the methyl 2,4,6-tri-O-methylgalactoside constituted ca. 23% of the methanolysate. The molar ratio of methyl 2,3,4,6-tetra-O-methylgalactoside, methyl 2,3,6-tri-O-methylgalactoside, and methyl 2,4,6-tri-O-methylgalactoside in the methanolysate was thus 1.31:0.76:0.60. These results suggest that this oligosaccharide is a mixture of 6-O-methyl-3-O-D-galactopyranosyl-2-O-methyl-D-galactose and 6-O-methyl-4-O-D-galactopyranosyl-2-O-methyl-D-galactose. On the other hand paper chromatography of the oligosaccharide indicated a single pure compound.

Although at present, no unique structure can be proposed for phyllymenan, it is fairly clear that the molecule is composed of 1,3- and β1,4-linked units. It can be seen that the experimental results indicate the presence of the following structural units in phyllymenan: β1,4-linked galactose, β1,4-linked 2-O-methyl-D-galactose, 1,3-linked galactose-2-

sulphate,/.....

sulphate, 1,3-linked galactose-2,6-disulphate, adjacent D- and L-galactose, and adjacent 6-O-methyl- and 2-O-methyl-D-galactose. It is obvious that phyllymenan and aeodan resemble each other in their gross structural features. On the other hand, phyllymenan differs from aeodan in having a higher proportion of monomethylgalactoses and a lower proportion of ester sulphate. In addition, phyllymenan is devoid of glycerol.

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