"A STRUCTURAL INVESTIGATION OF THE SULPHATED POLYSACCHARIDE OF *ANATHECA DENTATA* (SUHR) PAPENF. AND THE XYLAN OF *CHAETANGIUM ERINACEUM* (TURN.) PAPENF."

by

Irina Russell B. Sc. (Pharm.), B. Sc. (Hons.) (Rhodes).

A Thesis

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Department of Chemistry,
Rhodes University,
Grahamstown,
South Africa.

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SUMMARY

Hot-water extraction of Anatheca dentata, a red seaweed belonging to the family Solieriaceae, yielded a mixture of polysaccharides. Fractionation of this mixture with Cetavlon gave a glucomannan as minor component and a highly sulphated major component, which gave D- and L-galactose, D-xylose and small amounts of 3-O-methylgalactose, pyruvic acid and uronic acid on hydrolysis. All subsequent investigations were carried out on the sulphated major component. The sulphate was not labile to alkali, but was removed with methanolic hydrogen chloride. Periodate oxidation of the polysaccharide before and after desulphation indicated that new α-glycol groups were formed during desulphation. All the xylose units in the polymer were cleaved by periodate and this, together with the fact that the major xylose product from methylation analysis of the desulphated polymer was the 2, 3, 4-tri-O-methyl derivative, indicated that the xylose occurs as a non-reducing end-group. Methylation of the desulphated polysaccharide revealed the presence of 1, 4- and 1, 3- linked D-galactose and 1, 4- linked L-galactose units in the polymer. D-Glucuronic acid occurred as non-reducing end-groups. Partial acid hydrolysis and separation of the neutral components led to the isolation and characterisation of 4-O-β-D-galactopyranosyl-L-galactose (1), 3-O-α-L-galactopyranosyl-D-galactose (2), 4-O-β-D-galactopyranosyl-D-galactose (3), O-β-D-galactopyranosyl (1→4)-O-α-L-galactopyranosyl (1→3)-D-galactose (4), and O-β-D-galactopyranosyl (1→4)-O-α-L-galactopyranosyl (1→3)-O-D-galactopyranosyl (1→4)-L-galactose(5). This ideal series (1, 2, 4, 5) of oligosaccharides indicates that much of the macromolecule is composed of an alternating sequence of α-1, 3-D- and

β -1, 4-L-galactose residues. Separation of the acidic fragments of the partial acid hydrolysis led to the separation and characterisation of L-galactose 3-sulphate, L-galactose 6-sulphate, 4-O-α -D-glucuronosyl-L-galactose and glucuronic acid. Three pyruvic acid containing saccharides were also isolated: two of these were shown to be 4, 6-O(1′-carboxyethylidene)-D-galactose and 4-O- β - [4, 6-O (1′-carboxyethylidene)-D-galactopyranosyl] -L-galactose. The remaining one has so far not been characterised.

A xylan, isolated from the red seaweed, Chaetangium erinaceum, was shown by periodate and methylation studies to be essentially linear and to be composed of β -1, 3- and β -1,4 - linked units in the ratio 2:9. Partial, acid hydrolysis led to the isolation and characterisation of xylobiose, rhodymenabiose, xylotriose, O- β -D-xylopyranosyl (1→3)-O- β -D-xylopyranosyl (1→4)-D-xylose, O- β -D-xylopyranosyl (1→4)-O- β -D-xylopyranosyl (1→3)-D-xylose, xylotetraose, and xylopentaose.

1. INTRODUCTION

1. 1 Carbohydrate Sulphates

The widespread occurrence of carbohydrate sulphates in Nature is not always fully appreciated. In the plant kingdom, sugar sulphates are found combined in many polysaccharides of the algae,[1] although they are rarely found in land plants. In the animal kingdom, these esters occur in such mucopolysaccharides[2] as heparin, the chondroitins, and mucoitin sulphate; they are also found in the cerebron sulphate of the brain.[3]

From a chemical point of view, structure determination, especially of high-molecular-weight carbohydrate sulphates, presents a number of interesting problems and opportunities although at the same time, the sulphate group seriously handicaps structural investigations. Sulphate groups can normally be retained during methylation of the hydroxyl groups, and information about their location can therefore be obtained by subsequent hydrolysis. The information obtained is, however, ambiguous, and additional evidence is required to distinguish between the site of the sulphate and of glycosidic substitution. Unfortunately, it is extremely difficult, even under forcing conditions, to achieve complete methylation of a highly sulphated polysaccharide, presumably due to steric hindrance. Evidence for the location of sulphate ester groups and the modes of linkage between monosaccharide units may be obtained by various means, for example, by methylation of the desulphated polymer, periodate oxidation before and after desulphation, alkaline elimination, infrared spectroscopy, isolation of sugar sulphates after partial fragmentation, and measurement of the rate of hydrolysis of the sulphate ester.

The following review covers the more important aspects of the chemical and physical properties of sugar sulphates which are of value in the structural elucidation of sulphated polysaccharides. Part of this thesis, namely that concerned with the sulphated galactan from Anatheca dentata, illustrates the application of some of these methods.

1. 1. 1 Infrared Spectra

The infrared spectra of chondroitin sulphate isomers were examined by Orr,[4] who noted that, in addition to a band at 1240cm^{-1} attributable to the S-O bond-stretching vibration, another band at ca820 cm^{-1} could be attributed to a C-O-S vibrational mode. The exact position of the latter band was dependent on the spatial distribution of sulphate groups in the hexosamine moeities, being in an axial position on the sugar ring in one isomer (absorption at 855 cm^{-1}) and in an equatorial position in the other (absorption at 825 cm^{-1}). This frequency-structure correlation was confirmed by later work,[5] in which it was shown that sulphation of the equatorial primary hydroxyl group in D-galactose, D-glucose, and 2-acetamido-2-deoxy-D-glucose causes an absorption band at 820 cm^{-1}, and that the equatorial sulphate group in D-glucose 3-sulphate absorbs at 832 cm^{-1}.

After an examination of the spectra of a number of polysaccharide sulphates, it was suggested[5] that the appearance of a peak at 850 cm^{-1} was due to a sulphate group occupying an axial, secondary position, as, for example, on the hydroxyl group at C-4 of D-galactose when this sugar is in the usual C1 conformation.[6] This conclusion was subsequently verified when the spectrum of D-galactose 4-sulphate was obtained;[7] it has since been used as evidence for assigning a sulphate

group to the (axial) position at C-2 of L-rhamnose in a sulphated polysaccharide.[8]

However, it has been pointed out by Spedding[9] that other effects may also influence the position of the absorption maximum, particularly intermolecular forces in the solid state. For example, the difference (12 cm^{-1}) in the C-O-S frequencies of the 3-and 6-sulphate groups in the corresponding D-glucose monosulphates,[5] where both groups are equatorial may be due to intermolecular effects peculiar to the solid state in which the samples were examined, as was found to be the case for the axial and equatorial p-toluenesulphonate group in D-mannose derivatives.[10] Spedding suggested[9] that there is, possibly, also a change in the frequency of a rocking mode of the ring methylene group according to whether the group is adjacent to the ring oxygen atom, or not.

During studies of a number of synthetic sugar sulphates, Turvey *et al*[11] recorded the infrared spectra and the absorption maxima in the 800-860 cm^{-1} region of some galactose sulphates and their derivatives. The physical state of the specimen was found to often produce significant variations in the position of the absorption peak. This was particularly noticeable in the case of methyl α-D-galactopyranoside 2, 3-disulphate, where the major peak was at 833 cm^{-1} in a mull but was at 855 cm^{-1} in film. Considerable variations in the absorption maxima of D-galactose 4-sulphate and its derivatives were also apparent. Thus the free sugar barium sulphate had a major peak at 850 cm^{-1}, as expected for a pyranose ring with sulphate in an axial, secondary position, but also a shoulder at 835 cm^{-1} indicating the presence of other conformations or ring-forms in equilibrium. The methyl α-glycoside sulphate had a maximum at *ca* 815 cm^{-1}, suggesting either that the conformation of the pyranose ring

is not the expected C1 chair, or that some other factor is operative in determining the position of the peak. In the methyl tri-O- benzoyl-α-glycoside sulphate, the peak was again at 850 cm^{-1}, perhaps indicating a return to the expected conformation.

Harris and Turvey[12] observed that the principal absorption peaks in the region 800-870 cm^{-1}, for a range of sugar sulphates and glycoside sulphates, were independent of the cation present but dependent both on the physical state of the sample and on the presence of different aglycone groups and substituents. Extreme examples of the effect of the aglycone on the position of the C-O-S group vibration are methyl α-D-galactopyranoside 4-sulphate with a peak at 817 cm^{-1} compared with benzyl β-D-galactopyranoside 4-sulphate with a peak at 853 cm^{-1}, and methyl α-D-glucopyranoside 4, 6-disulphate (828 cm^{-1}) compared with D-glucose 4, 6-disulphate (855 cm^{-1}). The absorption band centred about 1250cm^{-1}, due to the S-O group vibration, shows some variability, but is too broad to be of diagnostic importance.

The 2-sulphates of D-glucose and D-galactose[13] both show absorption at higher frequencies than expected, particularly when in the form of syrups and this effect is more pronounced with the galactose isomer (850cm^{-1}). It is, of course, possible that the 2-sulphate may exist in some form other than the pyranose ring with the C1 conformation. Alternatively, factors other than the conformation may be of prime importance in determining the absorption maximum. That this is not due to a change in ring conformation from C1 (D) to IC(D) is apparent[12] from methyl 4, 6-O-benzylidene- α-D-glucopyranoside 2, 3-disulphate and the corresponding galactose compound, both of which are locked in the C1 (D) conformation by the benzylidene ring but still give a band near 850cm^{-1}.

Measurement of optical rotations and n. m. r. spectroscopy may both be used to study conformations in solution.[14] Except in the case of glycoside 4, 6-disulphates and 4, 6-O-benzylideneglycoside sulphates, the molecular rotations of aqueous solutions of a number of D-glucoside and D-galactoside sulphates have been shown[12] to differ very little from those of the parent glycosides. As the introduction of a sulphate group does not affect the molecular rotation of a glycoside, it is suggested that these sulphates all have the expected C1 (D) conformation in aqueous solution. The chemical shifts and coupling constants for the protons in a number of α-D-glucopyranoside and α- and β-D-galactopyranoside sulphates have been measured in aqueous solution.[15] The values recorded are in agreement with a C1 (D) conformation for both series of glycoside sulphates.

It is possible that, in the crystal lattice, some of the sugar sulphates assume conformations other than the expected C1 (D) form known to exist in solution for D-galactose and D-glucose. Only a crystallographic examination of these sulphates could finally settle this question.

Thus, the use of infrared spectra alone to assign positions to sulphate groups in carbohydrate sulphates requires some caution.

1. 1. 2 Acid Hydrolysis

Sugar sulphates, like most esters, are labile to both acids and alkalis. In view of the reported racemisation of optically active S-butyl hydrogen sulphate when treated with acid,[16] D-glucose 3-sulphate and D-galactose 4-sulphate were completely hydrolysed and the products examined.[17] The acid-catalysed hydrolysis of the sulphate-ester linkage proceeded with retention of configuration, the only sugar product

detected in each case being glucose and galactose, respectively. This behaviour is in keeping with the mechanism postulated for the acid-catalysed hydrolysis of steroid hydrogen sulphates,[18] which proceed by way of S-O bond scission and, hence, lead to retention of configuration on the carbon atom bearing the hemi-ester group.

Although certain sugar phosphates undergo phosphate-ester migration under acid conditions, sugar sulphates, when dissolved in dilute aqueous acid, do not appear to undergo sulphate-ester migration. For instance, when D-galactose 4-, D-galactose 6-, D-glucose 3- and D- glucose 6-(sodium sulphate) were separately heated at 100° in 0. 33N sulphuric acid, the only observable reaction was slow hydrolysis; no products of sulphate migration could be detected by paper chromatography.[19] However, it has been reported[20] that a migration of sulphate groups occurs in chondroitin sulphates when they are heated in the free acid form. When the free acid form of chondroitin 4-sulphate was heated, the absorption bands at 850cm^{-1} and 928cm^{-1} corresponding to the axial sulphate group,[21] disappeared, and a strong absorption band in the 820 cm^{-1} region, corresponding to an equatorial sulphate group,[21] appeared. The spectrum, thus, became apparently identical with that of chondroitin 6-sulphate. On the other hand, the spectra of the chondroitin 6-sulphate, which had been treated in a similar manner, were the same before and after heat was applied. Similar phenomena were observed for the oligosaccharides (free acid form) obtained from chondroitin 4- and 6-sulphate by testicular hyaluronidase digestion. This is in keeping with the generalisation that acyl migration occurs away from the anomeric centre.

During acid hydrolysis of glycoside sulphates, two concurrent

reactions take place, namely glycoside hydrolysis and sulphate-ester hydrolysis. A study of the hydrolysis of methyl β-D-galactopyranoside 6-sulphate[17] at various temperatures showed that with increase in temperature between 65° and 100°, the increase in the rate of glycoside hydrolysis was approximately the same as that of ester hydrolysis. This indicates that changes in reaction temperature do not appreciably alter the relative rates of hydrolysis of the two linkages. A more interesting effect is shown by a comparison of the glycoside hydrolysis in a glycoside sulphate with that in the parent glycoside. Thus the rate of hydrolysis of methyl β-D-galactopyranoside is several times faster than that for methyl β-D-galactopyranoside 6-sulphate, for methyl α- and β-D-glucopyranosides the rates are appreciably faster than for their 3-sulphates, but the effect is less pronounced than that shown by the galactoside 6-sulphate. It is apparent, therefore, that a sulphate group stabilises the glycosidic linkage and that a 6-sulphate shows a greater stabilising effect than a 3-sulphate. This effect seems to be general for any bulky substituent occupying an equatorial position on a pyranoid ring, and can be rationalised on conformational grounds.[17]

A further point of interest in this study[17] was the effect of a glycoside linkage on the rate of hydrolysis of the sulphate group. For methyl α-D-glucopyranoside 3-sulphate compared with glucose 3-sulphate, and for the galactoside 6-sulphate compared with galactose 6-sulphate, the effect was very small. Only in the case of methyl β-D-glucopyranoside 3-sulphate did the presence of a glycosidic linkage decrease appreciably the rate of hydrolysis of the sulphate group. The reason for this is not clear.

The rate at which sulphate groups are hydrolysed under acid conditions and, stemming from this, whether differences in the rate of hydrolysis can be used for assigning positions to the sulphate groups, is of particular interest in the field of polysaccharide sulphates. Acid hydrolysis studies on D-glucose monosulphates were carried out by Soda and Nagai.[22] They determined that the rate of hydrolysis of the sulphate group was in the order 3->4->6- sulphate. This result may be rationalized in terms of a generalization analogous to one well established in the cyclohexane series,[23] namely that esters of equatorial secondary hydroxyl groups are more rapidly hydrolysed than those of axial alcohols. For D-galactose sulphates and D-glucose sulphates, in the stable chair conformation of the pyranoid ring, such considerations apply, but, in addition, the primary hydroxyl group would be expected to form esters that would be relatively more stable than esters of the secondary hydroxyl groups. Other workers[24,25] have found that the differences in the velocity of acid hydrolysis for hexose and hexoside monosulphates, were insufficient to be useful for diagnostic purposes. Rees[26] has, however, developed hydrolysis conditions under which the position of attachment of the sulphate group can be determined in sugar sulphates and even in selected polysaccharide sulphates. He distinguished three groups of sulphates : (a) those with half-lives greater than 1.5 hours (when treated with 0.25 N hydrochloric acid at 100°), in which the sulphate group was on the primary hydroxyl group; (b) those with half-lives in the region of 1 to 1.5 hours (under the above conditions), in which the sulphate group was on a secondary, axial group; and (c) those with half-lives in the range of 0.1 to 0.4 hour, in which the sulphate group was on an equatorial, secondary hydroxyl group. Rees examined a number of polysaccharides containing more than one type of ester, and in each

case the relative amounts indicated by the hydrolysis curve were in accord with previous evidence, although the approximations necessary in the analysis of the kinetics did not allow any precise estimation of proportions. Thus fucoidin and the polysaccharide of Enteromorpha compressa each appeared from its hydrolysis curve to contain axial sulphate as the major component, with a smaller proportion of equatorial ester also present.

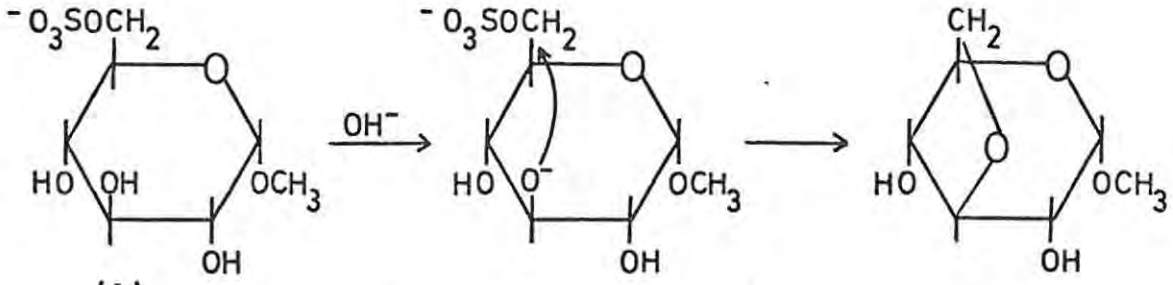
A direct comparison has been made of the hydrolysis of D-glucose 6- (potassium sulphate) and of potassium ethyl sulphate in 0.5 N hydrochloric acid.[27] Pseudo - first order rate constants were determined at three temperatures, and the energy of activation calculated for each compound from an Arrhenius plot. The activation energies were calculated to be 29 kcal.per mole for the sugar sulphate, and 30 kcal.per mole for the ethyl sulphate anion. The presence of the sugar ring appears to have little effect on the hydrolysis of the $-CH_2-OSO_3^-$ system, again suggesting S-O bond scission (rather than alkyl-O bond scission) as the rate-determining step.

1. 1. 3 Desulphation by Alkalis

The eliminations of carbohydrate sulphates in alkaline solution were elucidated by E. G. V. Percival[28] who found a close parallel between the behaviour of sugar sulphates and the analogous sulphonic esters.[29]

Di-O-isopropylidene -D-galactose 6-sulphate is not attacked by 2N sodium hydroxide solution during 6 hours at 100°.[24] This is not surprising since sodium alkyl sulphates are known to be relatively stable to hot aqueous alkali. However, many sugar sulphates yield all

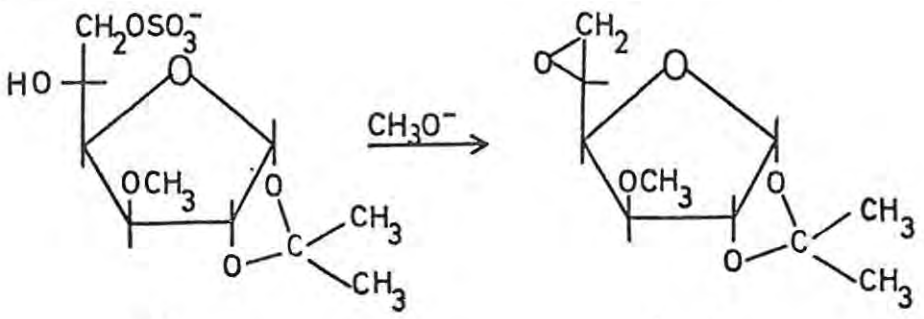
their sulphate in an ionisable form within 5 mins at 100° in 0.1N sodium hydroxide, but since disruption of the monosaccharide residues occurs this observation is of no diagnostic value. An investigation by Duff and Percival[30] established that the 6-sulphates of methyl α-and β-D-glucopyranosides and D-galactopyranosides (where C-1 is protected from alkaline degradation) gave the corresponding methyl 3,6-anhydro-hexosides on heating with alkali. Thus methyl α-D-glucopyranoside 6-sulphate (I) on treatment with alkali produces the 3,6-anhydrohexoside, provided that C-3 has a free hydroxyl group available. It was concluded, that sulphate



(I)

liberation with alkali proceeds only slowly, unless the removal of the sulphate group can lead to the production of an anhydro ring. The removal of sulphate is, therefore, a base-catalyzed elimination and not a hydrolytic process. Further evidence in support of this view was collected in the glucofuranose series.[31]

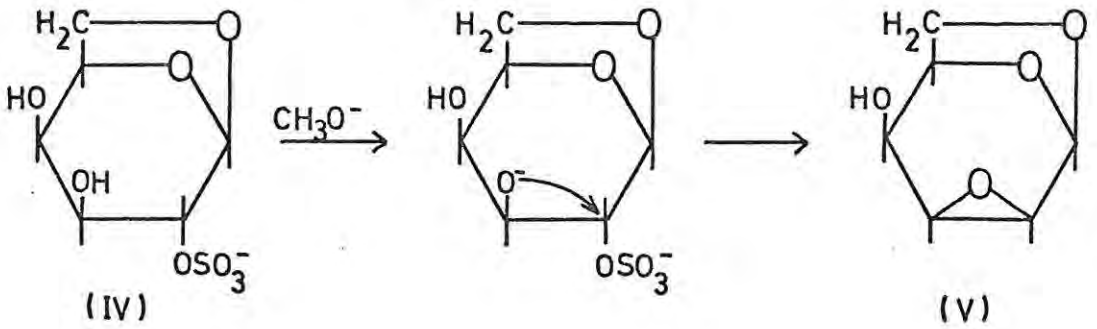
Reaction of 1,2-O-isopropylidene-3-O-methyl- α -D-glucofuranose 6-sulphate (II) with sodium methoxide at 40° readily gives the 5,6 anhydro derivative (III).[32]



(II) (III)

This type of anhydro ring may subsequently rearrange to a 3, 6-anhydrofuranoside if there is a free hydroxyl group on C-3. If neither C-3 nor C-5 bears a free hydroxyl group, hexoside 6-sulphates are relatively stable to alkali.

A sulphate group situated on a secondary hydroxyl group is alkali labile only if there is an adjacent trans free hydroxyl group present, the 1, 2 epoxide being formed. For example, 1, 6-anhydro- β -D-galactopyranose 2-sulphate (IV) forms 1, 6:2, 3-dianhydro - β -D-talopyranose (V),[33] when treated with sodium methoxide. Attack by the methoxide ion on either side and trans to the epoxide oxygen can then



occur with the formation of monomethyl sugars. No anhydride formation takes place when the sulphate ester group is cis to the neighbouring free hydroxyl group.

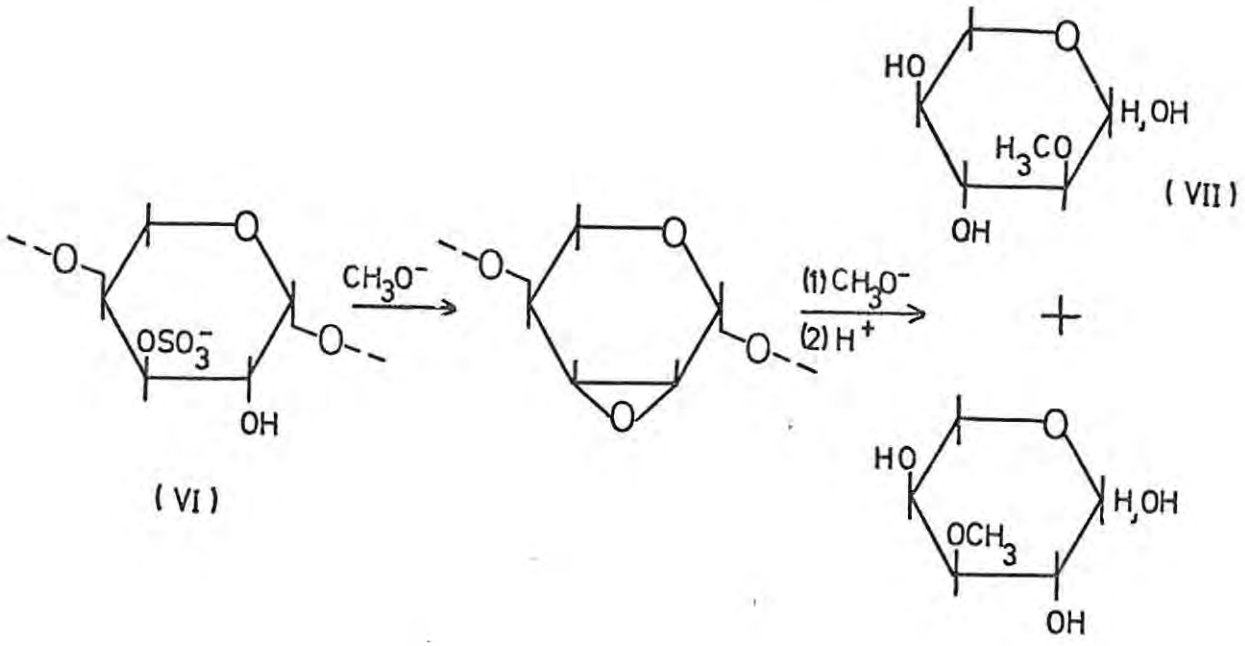
The above principles may be of value in assigning a position to a sulphate group on a glycoside and also in a sulphated polysaccharide. During early attempts[34, 35] to apply these principles in the polysaccharide field, precautions were not usually taken to avoid the release of sulphate by general destruction of the polysaccharide under the strong alkaline conditions, and the results gave at most a qualitative answer. Rees[36] subsequently devised a method whereby degradation can be eliminated and thus alkaline elimination can be exploited more fully for the structural analysis of some seaweed polysaccharides.

Porphyran, the galactan sulphate of the marine red alga Porphyra umbilicalis was used as a model because it contains L-galactose 6-sulphate units which are so linked that according to Percival's rules the ester should be labile to alkali, giving 3, 6-anhydro-L-galactose. In preliminary experiments, Rees found that desulphation proceeded at a convenient rate in N sodium hydroxide at 80°, but was attended by rapid destruction of the polysaccharide. Destruction of the polysaccharide was, however, substantially less when porphyran was reduced with potassium borohydride before the reaction, suggesting that the principal cause of degradation was "peeling" from the reducing end. The fact that slight destruction occurred even after reduction indicated a certain amount of depolymerization, probably exposing new reducing groups at which "peeling" could occur. This secondary degradation was avoided by including potassium borohydride in the actual reaction mixture, the new reducing groups presumably being protected under these conditions by immediate reduction.

It is of interest to note, that elimination of sulphate from the hydroxyl group at C-6 of suitable hexose derivatives leads to production of the 3, 6-anhydro derivative, together with a smaller amount of the parent hexose derivative.[30, 31] Thus, methyl β-D-galactopyranoside 6-sulphate on complete desulphation with alkali yielded the 3, 6-anhydro-D-galactopyranoside in a yield of about 78 per cent, the remainder being methyl β-D-galactopyranoside.[37] In contrast, Rees[36] showed that, in a polysaccharide sulphate, alkaline desulphation gave a quantitative conversion of suitably linked D-or L- galactose 6-sulphate residues into 3, 6-anhydro-D- or L-galactose residues.

Confirmation of the presence of ester sulphate groups on C-3 of the arabinose units of cladophoran[38] was obtained by treatment of the

polysaccharide with sodium methoxide followed by hydrolysis of the product and isolation of 2-O-methyl-L-xylose. The 2-O-methyl-L-xylose (VII) could only have arisen from 3-sulphated arabinose (VI) as shown in the reaction sequence below.



1. 1. 4 Other Methods of Desulphation

1. 1. 4. 1 Acetolysis

The sulphated polysaccharide is dissolved in absolute sulphuric acid and treated with a large excess of acetic anhydride at low temperatures, yielding the acetylated, desulphated product.[39] The reaction occurs without Walden inversion, since the desulphation of both D-glucose 3-sulphate and D-glucose 6-sulphate led to the isolation of α -D-glucopyranose pentaacetate, and thus probably involves electrophilic attack by the acetylium ion, $CH_3 C^+ = 0$, leading to scission of the

S-O bond. The reaction is, however, accompanied by significant glycosidic hydrolysis, the extent of which is dependent upon the nature of the glycosidic linkage, being negligible in the case of cellobiose and natural trehalose, but predominant in gentiobiose and melezitose yielding α-D-glucopyranose pentaacetate. Thus, this method of desulphation is of only limited use in the polysaccharide field.

1. 1. 4. 2 Methanolysis

Desulphation with methanolic hydrogen chloride has the advantage that scission of glycosidic linkages is virtually absent. Thus dry potassium chondroitin sulphate[40] when treated with 0. 06 M methanolic hydrogen chloride at room temperature yielded a non-dialysable, sulphate-free product with methylated carboxyl groups, with very little degradation. The method is not generally applicable since some sulphates, particularly hexose 6-sulphates in polysaccharides, are resistant to the reagent, leading to incomplete desulphation.[41] Also, in the presence of very acid-labile linkages, extensive depolymerization occurs.

Percival[42] found that whereas sulphate groups can be removed from carragheenin by the action of 1% methanolic hydrogen chloride leading to the isolation of 15% of degraded polysaccharide with a low sulphate content (1. 5%), similar treatment of fucoidin caused concomitant desulphation and degradation, and the recovered polysaccharide, isolated in low yield, had a slightly higher sulphate content than the starting material. However, when the water soluble polysaccharide of Enteromorpha compressa[8] (16% SO_4^{2-}) was kept for 48 hours with 0. 09M

methanolic hydrogen chloride, 71% of desulphated polysaccharide was recovered in which the sulphate content had been reduced to 0.75%. Comparison of the $[\alpha]_D$ and the percentage monosaccharide composition before and after desulphation, revealed that little if any change in the fundamental structure of the polysaccharide had occurred during the desulphation.

1. 1. 4. 3 Reductive Desulphation

Sulphate groups are not readily removed from sugar sulphates by catalytic hydrogenation and in fact tend to confer stability to catalytic hydrogenation on other groups present in a sugar derivative. Lithium aluminium hydride has been used[43] for the reductive fission of carbohydrate ester sulphate groups with the regeneration, in most cases, of the parent alcohol group; no deoxy sugars are formed. In this respect sugar sulphates differ from sugar p-toluenesulphonates since the parent alcohol group is regenerated by the action of lithium aluminium hydride on a secondary toluene-p-sulphonate, but a deoxy group is produced by reduction of a primary toluene-p-sulphonate.[44] Barium 1,2:5,6-di-O-isopropylidene-D-glucose 3-sulphate was reduced with lithium aluminium hydride in refluxing dioxan[43] to yield 1,2:5,6-di-O-isopropylidene-D-glucose. Reductive desulphation has to be carried out in very inert solvents such as ethers and dioxan, in which sugar sulphates are generally not very soluble. Alkali-metal borohydrides do not reductively cleave the sulphate group in di-O-isopropylidene-α-D-glucofuranose 3-sulphate, the only detectable reaction being a slow removal of the 5,6-O-isopropylidene residue, even at temperatures up to 100°.[45]

1. 1. 4. 4 Formation of Mixed Esters

Sugar sulphates and polysaccharide sulphates are insoluble in all but highly polar solvents, primarily as a result of the ionic, hemi-ester group, which complicates processes such as methylation and acetylation. For these reasons, attempts have been made to convert the hemi-ester into a diester with varied success. In all cases the resultant product was extremely unstable, decomposing even at room temperature. For example, Clancy[37] treated methyl α-D-glucopyranoside 3-(silver sulphate) with an excess of methyl iodide in dry methanol. After 16 hours, the silver iodide obtained corresponded to a 92% yield, but no diester could be isolated. A more successful preparation utilises ethereal diazomethane; an 80% yield of the methyl ester was obtained on treating a solution of di-O-isopropylidene- α -D-glucofuranose 3-(hydrogen sulphate) in methanol with diazomethane.[46] A similar procedure was used to convert O-methylated oligosaccharide and polysaccharide sulphates into the mixed esters. Mixed esters from methylated oligosaccharide sulphates were smoothly desulphated with lithium aluminium hydride, whereas the parent hemi-esters were unaffected by this reagent. This is, thus, a method of desulphation applicable at the oligosaccharide level and, possibly, also at the polysaccharide level.

1. 1. 4. 5 Reaction with Hydrazine

Hydrazine and semicarbazide have been used to effect desulphation of glucose and galactose sulphates. As yet, no general results can be postulated; the reaction is to a certain extent pH dependent, there is no decrease in the amount of hydrazine in solution during reaction,

and a free reducing end is required.[47] Other bases such as serine, L-glutamine, and imidazole do not catalyse the hydrolysis.[27] Disulphates are rapidly hydrolysed with the release of only one sulphate group, while glucose 6- and 3-sulphates and galactose 6-sulphate are hydrolysed only slowly.[48] It is possible that two sulphate groups on the same sugar residue are necessary for the occurrence of rapid hydrolysis although this has not been fully investigated. Aqueous hydrazine has no effect on chondroitin sulphates A and C but when barium chondroitin sulphate A is treated with anhydrous hydrazine,[49] a reagent which cleaves the amide linkage in, for example, proteins, a partially desulphated and highly (59-68%) N-deacetylated polymer is obtained.

1. 1. 4. 6 Enzymic Desulphation

Several sulphatases have been isolated from various sources. The sulphatase activities of preparations from marine molluses collected on the North Wales shore have been studied[50] with respect to various sulphated carbohydrates of both high and low molecular weight. Preparations from Calliostoma zizyphinum and Patella vulgata showed the widest range of activity, each liberating sulphate from D-glucose- and D-galactose-6-sulphate, chondroitin sulphate A and fucoidin. Other polysaccharides, keratosulphate, carrageenin and mucilages from Dilsea edulis and Porphyra umbilicalis were not desulphated. Littorina littorea and Mytilus edulis preparations contained glycosulphatase, but were inactive against chondroitin sulphate A and the algal polysaccharide sulphates. A chondrosulphatase has been obtained from Proteus vulgaris.[51]

Rees has isolated[52] an enzyme from Porphyra umbilicalis, which liberates sulphate from the ester linkages of porphyran with the simultaneous synthesis of 3, 6-anhydrogalactose units within the polysaccharide. The enzymic reaction is thus seen to be analogous to the well-known alkaline elimination of sulphate from hexose 6-sulphate derivatives. It is suggested that L-galactose 6-sulphate units in porphyran are converted, with fission of the C-O bond of the sulphate ester, into 3, 6-anhydro-L-galactose residues and that the enzyme is therefore not a true sulphatase. The enzyme shows diminished activity towards the products of partial acidic and enzymic hydrolysis of porphyran, and its action is not reversible.

1. 1. 5 Periodate Oxidation

The use of periodate oxidation in the structural investigation of carbohydrates is well established. Barry, Dillon, and their coworkers[35, 53] used this reagent in the structural investigations of a number of sulphated polysaccharides from algae and assumed that the sulphate groups are stable to periodate and behave as a simple blocking group akin, for example, to a methyl ether group. Support for this assumption comes from studies on the analogous sugar phosphates, in which the phosphate group appears to behave as a normal blocking group in neutral or slightly acid solution. Grant and Holt[45] have, however, reported that sulphate groups on some sugar sulphates are liberated on periodate oxidation but are stable in other cases. For example, when D-glucose 3-(sodium sulphate) was treated with 0. 35 M periodate for 7 days in the dark, no sulphate could be detected in the solution, but the reaction of D-galactose 6-(sodium sulphate) with 0. 35 M periodate under the

same conditions led to the liberation of all the sulphate as sulphate ions. In contrast, Turvey et al[54] have reported that no inorganic sulphate could be detected in the products of oxidation of either D-galactose 6-sulphate or D-glucose 6-sulphate, even after 216 hours at pH 8.2, with excess sodium metaperiodate.

Reducing sugar phosphates are usually considered as being oxidised in the ring form with production of intermediate formyl esters[55] but the consumption of periodate and end products of the reaction depend on the conditions of reaction and on the position of the phosphate group. It has been concluded from a study of the oxidation patterns of D-glucose 3- and 6-sulphates and D-galactose 3-, 4- and 6- sulphates[7, 13, 54] that the sugar sulphates are oxidised in the pyranoid ring forms. Thus, when D-glucose 6-sulphate[54] was oxidised by periodate in an unbuffered solution, there was an initial rapid (1 hour) consumption of over 3 moles of periodate with the liberation of 2 moles of formic acid, the latter rising to 3 moles after a few hours, and finally both the periodate consumption and the formic acid liberation became constant at about 3.5 moles. However, Suzuki and Strominger[56] reported that the oxidation patterns for the 4- and 6- sulphates of 2- acetamido -2-deoxy-D-galactose suggested that these compounds were oxidised in the open-chain forms. It has also been suggested[13] that the oxidation of D-galactose 2-sulphate and D-glucose 2-sulphate proceeds via the acyclic form of the sugar sulphate. The faster initial rate of oxidation for the galactose isomer compared with that of the glucose isomer may reflect a greater equilibrium concentration of open-chain form in the galactose series than in the glucose series.

Not enough monomeric glycoside sulphates have been examined to permit more than a few preliminary observations to be made.

Methyl β-D-galactopyranoside 6-sulphate consumed[54] 2 moles of periodate per mole within 30 minutes and thereafter underwent little further oxidation, in agreement with predicted values. Methyl α-D-glucopyranoside 6-sulphate has a similar oxidation pattern.[57] Since polysaccharide sulphates resemble glycoside sulphates, rather than free sugar sulphates, the use of periodate oxidation in structural studies would appear to be justified, although examination of more glycoside sulphates is desirable before an unequivocal answer can be given.

Periodate oxidation of sulphated polysaccharides, before and after desulphation, has been used to assign the position of ester sulphate groups, for example in the case of Ulva lactuca.[58] It is known that in buffered solution at low temperature, vicinal cis-hydroxyl groups in sugars are selectively oxidised by periodate. Since in buffered solution at 2° the partially desulphated polysaccharide from Ulva lactuca reduced twice as much periodate as the sulphated polysaccharide, it was concluded that desulphation furnishes cis- rather than trans-glycol groupings. L-Rhamnose is the only sugar present in the polysaccharide which has cis-glycol groupings. These occur at positions-2 and -3 and it was assumed therefore that sulphate groups are linked either to C-2 or C-3 in rhamnose. On infrared spectroscopic evidence, the sulphate groups were tentatively assigned to position-2 of the rhamnose residues.

1. 2 Xylans from Red Algae

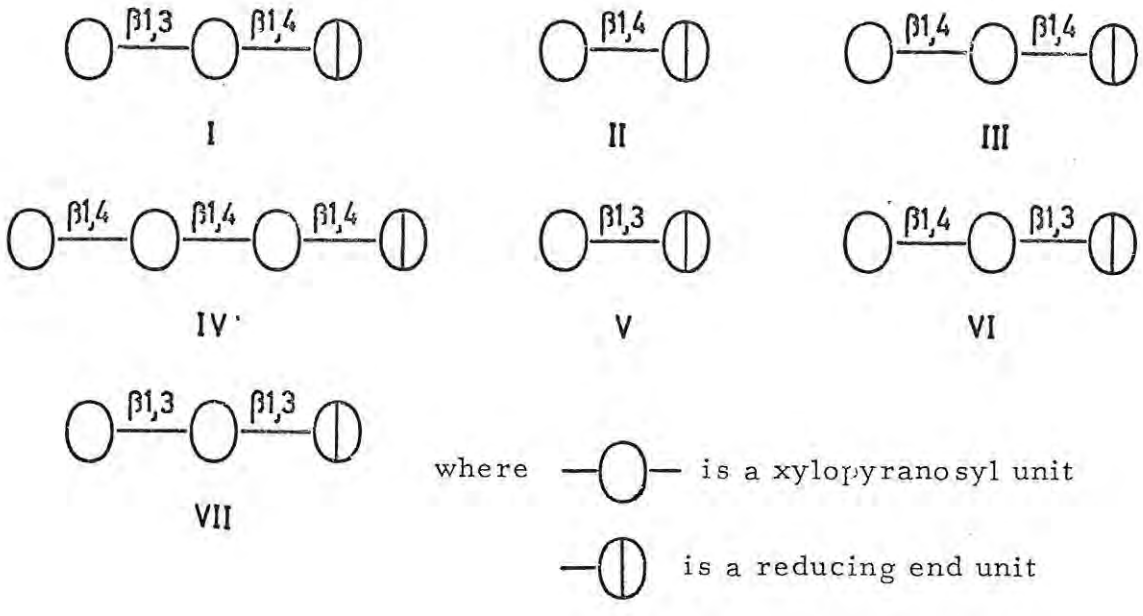
From a study of the chief structural features of some xylans from red algae, Turvey and Williams[59] have recently suggested that xylose occurs in these algae in at least two distinct types of structure; first in the form of either a separate xylan or combined in a heteropolysaccharide, neither of which is a skeletal material, and in which both 1, 3- and 1, 4-linkages occur; secondly as a linear polysaccharide in the cell wall, in which it is either completely 1, 3-linked, or completely 1, 4-linked. In contrast, xylans from most land plants, for example esparto xylan,[60] consist of linear chains of β-1, 4-linked D-xylopyranose units, to which may be attached side chains of arabinose units or other sugars, and xylans in the cell walls of certain siphonaceous green algae have been shown to consist of linear chains of β-1, 3-linked D-xylopyranose units.[61, 62]

The water soluble xylan from the red seaweed, Rhodymenia palmata, was first isolated by Barry and Dillon.[63] Periodate oxidation studies[64] showed that 80% of the xylose residues possess α-glycol groupings and these are linked, therefore, through C1 and either C2 or C4, if the possibility of the presence of furanose residues is discounted. The resistance to attack of the remaining 20% can be explained only by the presence of 1, 3-linkages. Complete methylation and hydrolysis gave 2-O-methyl-, 2, 3-di-O-methyl-, 2, 4-di-O-methyl- and 2, 3, 4-tri-O-methyl-D-xylose. This evidence suggests that the xylan is branched and contains both 1, 3- and 1, 4- linkages. The molecular size of the xylan was estimated at 39-40 xylose units[65] with, on average, one branch point in each molecule.

Evidence obtained from the Barry[66] degradation indicated that the polysaccharide is homogeneous and not a mixture of 1, 3- and 1, 4-linked

xylans. These findings were supported by Howard[67] who isolated, in substantial yield, a pure crystalline trisaccharide containing both a 1,3- and a 1,4-linkage, from partial hydrolysis of the xylan by ruminal micro-organisms. The trisaccharide was identified as O- β -D-xylopyranosyl (1→3)-O- β -D-xylopyranosyl (1→4)-D-xylose (I) and control experiments showed that the bacterial suspension did not produce oligosaccharides containing β-1, 3 - xylosidic linkages by trans-β - xylosylation.

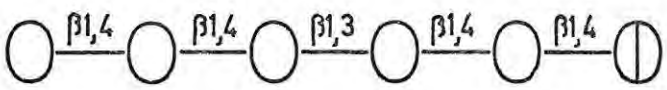
Application of the Smith degradation[68] to the xylan from Rhodymenia palmata indicated that a small proportion of adjacent 1,3-linked xylose residues are located in the xylan chain, although the major portion of 1,3-linked xylose residues are flanked by 1,4-linked residues. These results were confirmed by degradation of the xylan with an enzyme preparation from Myrothecium verrucaria. The



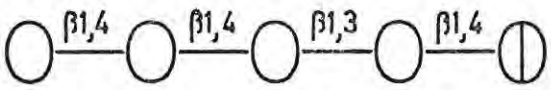
products included oligosaccharides I→V, and trace amounts of two trisaccharides tentatively identified as having structures VI and VII.

Jensen and coworkers[69] observed that stepwise extraction of dried Rhodymenia palmata, first with water and then with 0.2 N hydrochloric acid, yielded two fractions, of which the former gave considerably more viscous solutions than the latter. They suggested that the two fractions are chemically similar but differ in molecular weight. Two fractions of the xylan, one obtained by extraction with water (xylan A_1) and the other by subsequent extraction of the residue with 0.2N sulphuric acid (xylan B), have been investigated by methylation analysis.[70] Small portions of the methylated polysaccharides were subjected to methanolysis and the relative proportions of 2,3,4-tri-, 2,3-di- and 2,4-di-O-methyl-D-xylose were determined by gas-liquid chromatography and found to be 2:72:26 for xylan A_1 and 3:57:40 for xylan B. These ratios agreed fairly well with the values estimated by periodate oxidation. The amount of mono-O-methyl-D-xyloses was low and was considered to have resulted from undermethylation and demethylation during the hydrolysis. It was concluded, therefore, that both xylans are unbranched but have slightly different proportions of β-1,4- and β-1,3-linkages.

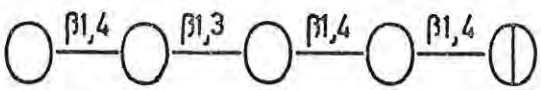
A third xylan fraction (xylan A_2), obtained by extraction with water, was hydrolysed using a specific enzyme. The oligosaccharides VIII→XII, all of which contain one β-1,3-linkage, were isolated and characterised.



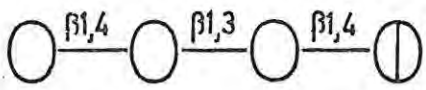
VIII



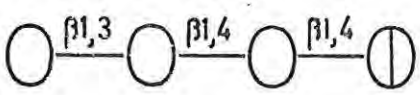
IX



X



XI



XII

Since it appears that the enzyme used for the partial hydrolysis preferentially attacks β-1,4-linkages, when these are flanked by other β-1,4-linkages, Björndal et al deduced that the β-1,4- and -1,3-linkages in the polysaccharide are not regularly arranged and that a purely random arrangement of the residues seems more probable. The formation of the tetrasaccharide XII is not in agreement with the assumed requirements of the enzyme. One possibility is that it is formed from the non-reducing end of some polymers of appropriate structures. On the other hand, it is possible that the restrictions assumed for the action of the enzyme are not rigorous.

The method of sequential extraction of dried Rhodymenia palmata was recently used[59] to give a series of xylan fractions which were subjected to methylation analysis (Table A). These results indicate that at least two distinct xylans are present in the alga. Sequential extraction of this alga with cold dilute acid, with hot water, and with cold dilute alkali gave three xylan fractions which differ very little in composition. Each is essentially linear and contains 1,3- and 1,4-linkages in a ratio varying from 1:4 to 1:5. In contrast, extraction of the residual weed with hot 3N alkali (under N_2) gave a linear xylan with almost exclusively 1,4-linkages. It is probable that this xylan is derived from the skeletal material of the cell wall, although not necessarily from the microfibrils. In the investigations of Cronshaw et al[71] the microfibrils of the Rhodymenia palmata cell wall were found, by hydrolysis and paper chromatographic analysis of the resulting sugars, to contain approximately equal quantities of glucose and xylose residues. The xylose units were assumed, on the evidence of X-ray diffraction analysis and electron microscopy, to be 1,3-linked in either a xylan or in a xyloglucan.[72]

TABLE A: Molar % O-methylxyloses from Rhodymenia palmata

Extracting Solvent	2-O-methyl-xylose	2,3-di-O-methyl-xylose	2,4-di-O-methyl-xylose	2,3,4-tri-O-methyl-xylose
0.5N HCl	1.2	80.3	16.4	2.1
H_2O	2.5	75.1	18.6	3.8
N NaOH	1.65	77.6	17.5	3.25
3N NaOH	2.2	94.4	2.1	1.3

The red alga, Porphyra umbilicalis, has been shown to contain a xylose-rich fraction, extracted from the cell wall by alkali. The residue, after extensive hot-water extraction of the alga, yielded two polysaccharide fractions when extracted with cold N sodium hydroxide followed by hot 20% sodium hydroxide.[73] Both fractions gave mannose, xylose, and glucose on hydrolysis but they differed in that mannose was the main constituent of the dilute alkaline extract while xylose was more abundant in the extract with concentrated alkali. Separate X-ray diffraction analysis of the cuticle and the cell wall of Porphyra[74] has shown these to be markedly different. The cuticle, covering the whole membranous thallus of the plant, is not microfibrillar and consists mainly of mannan. The cell wall proper, on the contrary, contains abundant microfibrils constructed of β-1,3-linked xylan. In agreement with these conclusions, Turvey and Williams,[59] obtained a pure xylan from Porphyra umbilicalis by extraction with alkali. Methylation analysis of this xylan, which is considered to be microfibrillar in origin, showed it to be completely 1,3-linked.

The chlorite extract of Porphyra umbilicalis,[59] after removal of

the water soluble polysaccharides, has also been shown to contain a xylan. This xylan, however, contains both 1, 3- and 1, 4-links in a ratio of about 1:5. 5 with a small amount of branching as judged by the yield of 2-O-methylxylose. A xylan very similar to that of the chlorite extract of Porphyra has been obtained from Laurencia pinnatifida by extraction with alkali.[59]

X-ray diffraction analysis of the cell wall and cuticle of Bangia fusco-purpurea,[74] a marine alga closely related to Porphyra, showed the presence of both crystalline mannan and crystalline xylan. There is no doubt that the cell wall of Bangia is also constituted of β -1, 3-linked xylan.

Two very similar xylan fractions, obtained from Rhodochorton floridulum by hot-water extraction and chlorite treatment, have been examined by the methylation technique.[59] (Table B.) These results

TABLE B: Molar % O-methylxyloses from Rhodochorton floridulum

Extracting Solvent	2-O-methyl-xylose	2, 3-di-O-methylxylose	2, 4-di-O-methylxylose	2, 3, 4-tri-O methylxylose
H_2O	11. 1	53. 7	14. 1	21. 1
Chlorite	11. 1	38. 9	10. 9	39. 2

show a ratio of 1, 3- to 1, 4-links in the region of 1:4 but also indicate a considerable degree of branching. Furthermore, the high yields of tri-O-methylxylose compared with 2-O-methylxylose strongly suggest that the xylose chains are short side branches on another polysaccharide. Since it was found to be impossible to isolate a xylan completely free from other sugars, it was tentatively suggested by Turvey and Williams that the

fractions from Rhodochorton may be heteropolysaccharides in which the xylose occurs as short, branched chains on another polysaccharide backbone (probably a glucan). The recognition of 2, 4, 6- tri-O-methylglucose as another product of methylation of these fractions suggests that a 1, 3-linked glucan is present.

The hot-water extract of Chaetangium fastigiatum, a red seaweed which belongs to the same order as Rhodochorton floridulum, namely the the Nemalionales, has recently been examined.[75] The main water-soluble polysaccharide is a xylan which showed a single peak in the ultracentrifuge, broad enough to suggest polydispersity. Graded precipitation by the addition of ethanol to an aqueous solution gave fractions which differed in optical rotation and in behaviour towards periodate oxidation. The major fraction (80%) had $[\alpha]_D$ -112° and periodate oxidation studies indicated that ca 85% of the xylose units are linked β-1, 4- or β-1, 2-, if xylofuranose residues are absent. Methylation of the total xylan, followed by hydrolysis, led to the isolation of 2-O-methyl-, 2, 3-di-O-methyl-, 2, 4-di-O-methyl- and 2, 3, 4-tri-O-methyl-D-xylose in the approximate molar proportions 1:62:19:1.4. The polysaccharide therefore contains chains of β-1, 3- and β-1, 4-linked residues, has an average chain-length of 50-60, and appears to be branched to a small degree.

Xylose is a known constituent of many other polysaccharides from red algae but its mode of linkage has seldom been determined. In those cases where the mode of linkage has been established, there is often uncertainty as to whether the xylose occurs as a separate xylan or as part of a heteropolysaccharide.

2. EXPERIMENTAL

Paper chromatography was carried out on Whatman No. 1 filter paper using the following solvent systems: (A) ethyl acetate-acetic acid-formic acid-water (18:3:1:4), (B) ethyl acetate-pyridine-water (8:2:1), (C) butyl alcohol-ethanol-water (40:11:9), and (D) ethyl acetate-pyridine-water (10:4:3). Spray reagents used were (1) p-anisidine hydrochloride,[76] (2) periodate-benzidine,[77] (3) bromocresol green[78] (0.1% solution in 95% aqueous ethanol made just alkaline with sodium hydroxide) and (4) 20% sulphuric acid in ethanol. R_{Gal} and R_{Xyl} values refer to rates of movement relative to those of galactose and xylose, respectively. Electrophoresis was carried out on Whatman No. 1 paper using 0.4M sodium tetraborate buffer (pH10) at 50mA. M_X values refer to rates of movement relative to that of xylose. The degree of polymerisation (DP) of oligosaccharides was determined by the phenol-sulphuric acid method.[79] Infrared spectra were recorded on a Beckman IR-8 spectrophotometer. Concentration of solutions was carried out at 40°/20 torr and specific rotations were measured in water, unless otherwise stated. Sulphate was determined with 4-chloro-4′-aminodiphenyl.[80]

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. R_{TMG} values of methylated sugars refer to rates of travel relative to that of 2,3,4,6-tetra-O-methyl-D-galactose. Gas-liquid chromatography (g.l.c.) was carried out on a Beckman GC-4 chromatograph equipped with dual flame-ionisation detectors and nitrogen as carrier gas using the following columns: (a) 15% w/w butan-1,4-diol succinate polyester, on acid-washed Celite (80-100 mesh) at 175° for methyl glycosides; (b) 20% w/w Apiezon M on

Chromosorb W (80-100 mesh; acid-washed and treated with dimethyl-chlorosilane) at 200° for glycitol acetates; and (c) 3% w/w ECNSS-M on Chromosorb W (100-120 mesh) at 180° for acetylated nitriles, unless otherwise stated. Retention times T, T_E and T_X are relative to those of methyl 2, 3, 4, 6-tetra-O-methyl- β -D-glucopyranoside, tetra-O-acetylerythritol and 3, 5-di-O-acetyl-2, 4-di-O-methyl-D-xylononitrile, respectively.

2. 1 Anatheca dentata

2. 1. 1 Extraction and purification of polysaccharide

Wet Anatheca dentata (5. 6 kg) was mascerated, mixed with hot-water, and the pH adjusted to 3 with glacial acetic acid. Steam was passed into the mixture for 0. 5 h with constant stirring, after which the solution was strained through muslin and centrifuged hot, yielding a clear, pale-pink mucilage. When the extract was set aside for 24 h at 4°, a colloidal precipitate appeared which was removed by centrifugation. Precipitation into ethanol (5 vol.) afforded a fibrous, white product which was washed with ether, and dried (280g; 5% on a wet wt. basis). Purification of the polysaccharide was effected by dissolution in water, centrifugation of the solution, and precipitation into ethanol (5 vol.). The recovered polysaccharide was washed with ether and dried in vacuo at 45°. [Found (on material dried at 70°/0. 5 torr): N, 0. 53; SO_4^{2-}, 30. 5%] . The polysaccharide failed to precipitate from solution on the addition of potassium chloride solution and in this respect resembles the λ-fraction of carrageenan.[81] The polymer did not precipitate from solution when treated with Fehling's solution.

Chromatographic examination (solvents A, B, and C) of a neutralised acid hydrolysate revealed spots corresponding to galactose (major), xylose, and traces of two other sugars having R_{Gal} 2. 1 (yellow) and 2. 91 (yellow) (solvent B) with spray 1. Spray 3 revealed the presence of a small amount of uronic acid.

2. 1. 2 Separation and characterisation of the components of the polysaccharide

Polysaccharide (6. 0g) was hydrolysed with N sulphuric acid (50 ml)

for 16 h on a boiling-water bath. After neutralisation with barium carbonate, the solution was evaporated, to a partially crystalline syrup (4.45g) and applied to a cellulose column (50 x 5.4 cm) which was eluted with butyl alcohol-water (95:5). Fractions (ca 25 ml) were collected and, on the basis of paper chromatography, combined into three fractions which were evaporated to dryness.

Fraction I. The syrup (0.59 g) was shown by paper chromatography to contain xylose, and traces of glucose, mannose, arabinose, and two other sugars having R_{Gal} 2.1 and 2.91 (solvent B). The syrup was decolourised with charcoal in water, filtered, and evaporated to dryness. Recrystallisation of the residue from ethanol yielded crystals (120 mg), m.p. and mixed m.p. 143-144° with authentic D-xylose, $[\alpha]_D^{23}$ + 45° (6 min) ⟶ + 18.9° (c 0.5). The O-dibenzylidene dimethyl acetal derivative[82] had m.p. and mixed m.p. 212-213° (Kofler hot stage) with an authentic sample.

Fraction II. The syrup (0.58 g) was shown by paper chromatography (solvents A and B) to contain galactose (major sugar), xylose, glucose (trace), mannose (trace), and a sugar having R_{Gal} 2.1 (solvent B) (trace).

Fraction III. A portion of this fraction (3.05 g) was recrystallised from ethanol, giving galactose, m.p. 161-162°, $[\alpha]_D^{22}$ + 17.7° (c 0.50). The value obtained for the specific rotation indicated a D:L-galactose ratio of 1.57:1. Oxidation with nitric acid-water (1:1) yielded mucic acid, m.p. and mixed m.p. 212-213° with authentic mucic acid.

2.1.3 Fractionation of polysaccharide with Cetavlon

Polysaccharide (1.0 g) in water (50 ml) to which a solution of

cetyltrimethylammonium bromide (Cetavlon; 15 ml; 10% w/v solution) had been added was allowed to stand overnight. The precipitated complex was centrifuged and washed four times with water. The supernatant liquid was retained for further investigation (see later). The complex was redissolved by stirring overnight in 4M potassium chloride solution (50 ml). The insoluble residue was removed by centrifugation and discarded, and the clear solution precipitated into ethanol (5 vol.). The precipitated polysaccharide was washed with ethanol and then redissolved in water (50 ml), dialysed against running water, concentrated, and reprecipitated into ethanol (5 vol.). The polysaccharide was washed with ethanol and finally ether, and dried at 50° in a vacuum (0.73 g). Chromatographic examination of an acid hydrolysate (solvents A and B) showed the presence of galactose (major), xylose, traces of two other sugars R_{Gal} 2.1 and 2.91 (solvent B), and a minute trace of glucose. To the solution remaining after removal of the precipitated complex, was added sufficient potassium iodide to precipitate the excess Cetavlon. After removal of the Cetavlon - iodide complex by centrifugation, the supernatant was dialysed against running tap water, concentrated and freeze-dried to an off-white foam (31 mg). Chromatographic examination of an acid hydrolysate (solvent B) showed the presence of glucose and mannose.

All subsequent experiments were performed on the Cetavlon-precipitated polysaccharide. Further purification of the polysaccharide for analysis was effected by repeated (twice) dissolution in water, centrifugation of the solution, and precipitation in ethanol (5 vol.). After collection in a centrifuge, the polysaccharide was washed with ether, and dried in a vacuum at 45°, $[\alpha]_D^{25}$ -40.5° (c 0.99) [Found (on material dried at 60°/0.5 torr): 3,6-anhydrogalactose,[83] 0.41; OMe,

0.00; N, 0.0; SO_4^{2-}, 35.5%; equiv. wt. (from SO_4^{2-} detn.), 271]. The infrared spectrum (KBr disc) of this polysaccharide is given in Fig. 2.

2. 1. 4 Quantitative estimation of sugar residues in the Cetavlon-precipitated polysaccharide

The sugar residues in the polysaccharide were quantitatively estimated using a gas chromatographic method devised by Bowker and Turvey[84] based on the procedure of Gunner, Jones and Perry.[85] Dry polysaccharide (103.13 mg) and erythritol (10.00 mg) in N sulphuric acid (3 ml) were heated at 100^0 for 3 h. The mixture was neutralised with barium carbonate, centrifuged and the supernatant solution treated with sodium borohydride (20 mg) for 18 h. The solution was neutralised with N sulphuric acid, evaporated, and dried in vacuo at 30^0. To the dried mixture was added acetylation mixture (2 ml; 50:1, acetic anhydride: sulphuric acid) and the flask was stoppered and kept at 80^0 for 6 h. The mixture was then cooled, diluted with water (2 ml) and cautiously neutralised to pH5 with sodium bicarbonate. The glycitol acetates were extracted with chloroform (2x20 ml), the combined chloroform extracts were dried (magnesium sulphate) and after suitable concentration 1 µl samples were injected into the gas chromatograph. The concentration of a given sugar was estimated from the peak area of its glycitol acetate (mean of three injections) compared with that of the internal standard, erythritol acetate. Glycitol acetates do not give a molar response in the detector and thus standard curves were prepared by treating each sugar plus the internal standard (67.15 mg galactose and 10.42 mg erythritol; 26.70 mg xylose and 10.90 mg erythritol) with sodium borohydride and then subjecting the mixture to the above procedure.

2. 1. 5 Attempted fractionation of Cetavlon - precipitated polysaccharide by chromatography on DEAE - Sephadex A-50

The anion exchange gel DEAE-Sephadex A-50 was swollen and equilibrated with a solution of 0. 1M potassium chloride before being packed in a column (30 x 4. 5 cm). Polysaccharide (200 mg) in water (14 ml) was applied to the column, which was then sequentially eluted with the following solutions: (i) 0. 75M potassium chloride (2. 8 l), (ii) 1. 0M potassium chloride (2. 8 l), and (iii) 1. 5M potassium chloride (1. 0 l). Fractions (20 ml each) were collected and analysed for carbohydrate with the phenol-sulphuric acid reagent.[86] A plot of optical density against fraction number is shown in Fig. 1. Three distinct fractions were obtained, the solutions of which, on dialysis, evaporation and freeze-drying gave fraction 1 (46 mg), fraction 2 (112 mg) and fraction 3 (14. 5 mg). These fractions were eluted with potassium chloride solutions Numbers i, ii, and iii respectively. No further material was recovered from the column by increasing the concentration of potassium chloride. The properties of fraction 2 (major fraction) and those of the Cetavlon-precipitated polysaccharides are compared in Table I. The sugar residues of fraction 2 were quantitatively estimated as outlined earlier (see 2. 1. 4).

2. 1. 6 Isolation of pyruvic acid (as the 2, 4-DNP derivative) from polysaccharide

Polysaccharide (5. 0 g) was heated on a boiling-water bath with N sulphuric acid (20 ml) for 9 h. After neutralisation (barium carbonate) and centrifugation, the solution was shaken with Amberlite IR-120 (H^+) resin. The resin was filtered off and the acidic solution extracted with ether

(400 ml; in portions). The ether was removed by distillation and a small portion of the residue refluxed with 3% methanolic hydrogen chloride for 3 h. The solution was neutralised (lead carbonate), filtered, and on g. l. c. examination showed a peak having a retention time identical with that of methanolysed pyruvic acid (column (c) at an operating temperature of 100° and a nitrogen flow rate of 25 ml/min). This peak was identical with that of methanolysed furfural on columns (a) and (b) but was separated from it on column (c) under the above conditions (Retention times on column (c): methanolysed pyruvic acid 2. 1 min, methanolysed furfural 1. 9 min).

The remainder of the ether extract was treated with a methanolic solution of 2, 4-dinitrophenylhydrazine and the resultant mixture of 2, 4-dinitrophenylhydrazones separated by preparative t. l. c. using ethyl acetate as solvent. The appropriate portions of the plates were scraped off, extracted with methanol, treated with Amberlite IR-120 (H^+) resin and evaporated to dryness (12 mg). The residue crystallised from ether and had m. p. and mixed m. p. 218-219° with pyruvic acid 2, 4-dinitrophenylhydrazone. The infrared spectrum of this material (KBr disc) was identical with that of the authentic pyruvic acid derivative.

2. 1. 7 Action of alkali on polysaccharide

Polysaccharide (1 g) in water (150 ml) containing sodium borohydride[36] (0. 2 g) was set aside for 48 h at room temperature. Sodium hydroxide (7 g) and sodium borohydride (1 g) were then added, and the mixture was maintained at 82° ± 2°. After 4. 5 h, a further amount of sodium borohydride (1 g) was added, and, after 10. 5 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 (0.76 g), $[\alpha]_D^{20}$ - 36.4° (c 0.66) [Found: SO_4^{2-}, 35.3; 3,6-anhydrogalactose,[83] 0.70%]. A portion of the product (0.40 g) was subjected to a second treatment with alkali as above, giving a white solid (0.38 g), $[\alpha]_D^{20}$ - 33° (c 0.61) [Found: SO_4^{2-}, 34.8; 3,6-anhydrogalactose, 1.31%]. Chromatography of a neutralised, acid hydrolysate (solvents A and B) revealed the presence of galactose and xylose.

2.1.8 Treatment of polysaccharide with sodium methoxide

To polysaccharide (0.5 g) in water (25 ml) was added sodium borohydride (0.1 g). The mixture was allowed to stand for 24 h with occasional shaking, after which time further sodium borohydride (0.1 g) was added and the solution left for another 24 h. The polyalcohol was dialysed against frequently changed distilled water, concentrated, and isolated by freeze-drying. The polyalcohol (dried at 60° over P_2O_5 in *vacuo* for 24 h) was added to a solution of sodium (1.5 g) in absolute methanol (70 ml) and the mixture was refluxed for 24 h in an atmosphere of nitrogen. The insoluble material, after centrifugation and washing with methanol, was dissolved in water (50 ml) and dialysed against frequently changed distilled water for 3 days. Concentration of the solution followed by freeze-drying yielded a white foam (0.43 g). Chromatography of a neutralised, acid hydrolysate (solvents A and B) showed the presence of galactose and xylose. No artefacts could be detected.

2. 1. 9 Methylation of polysaccharide

To polysaccharide (10. 0 g) dissolved in the minimum quantity of water (50 ml) was added sodium hydroxide solution (40% w/w; 200 ml). The solution was stirred vigorously in an atmosphere of nitrogen, and dimethyl sulphate (100 ml) was added over 0. 75 h. Four similar additions of sodium hydroxide solution and dimethyl sulphate were made at 2 hourly intervals, at the end of which time the mixture was dialysed against running tap water (7 days). The solution was concentrated to a small volume. Hydrolysis of a portion of this solution (N sulphuric acid at 100° for 16 h), followed by neutralisation with barium carbonate and subsequent paper chromatography, revealed the presence of a large amount of galactose. Ten further additions of the above reagents were made to the partially methylated polysaccharide. The mixture was then dialysed, concentrated and the product isolated by freeze-drying (12. 50 g) [Found: OMe, 9. 69%] .

To a portion (2 g) of the partially methylated polysaccharide dispersed in dimethyl sulphoxide (150 ml), was added powdered sodium hydroxide (20 g) and dimethyl sulphate (10 ml; over 5 min) with constant stirring. Further additions of these reagents were made after 1 h and 24 h. The mixture was stirred for another 16 h, dialysed against running tap water (7 days), concentrated and the product isolated by freeze-drying (1. 78 g) [Found: OMe, 10. 15%] . The infrared spectrum (KBr disc) of this product showed a large hydroxyl peak. Further treatment with the above reagents failed to increase the methoxyl content.

2. 1. 10 Desulphation of polysaccharide[8]

Polysaccharide (1. 0 g) was shaken with 0. 1 M methanolic hydrogen

chloride (75 ml) for 48 h at room temperature, after which the undissolved polysaccharide was removed and washed with dry methanol. It was then dissolved in water (50 ml) and dialysed against frequently changed distilled water for 3 days. Concentration and freeze-drying of the solution gave a partially desulphated polysaccharide (0.72 g; 85%) [Found: SO_4^{2-}, 23.4%]. The supernatant methanolic solution, after neutralisation (silver carbonate) and concentration, gave a non-reducing syrup (282 mg). Paper chromatography of a neutralised, acid hydrolysate revealed the presence of galactose and xylose (solvents A and B).

Increasing the strength of the methanolic hydrogen chloride to 0.15M resulted, after a single treatment (48 h), in an 87% yield of polysaccharide. [Found: SO_4^{2-}, 19.9%].

In a third experiment, polysaccharide (1.0 g) was shaken with 0.1 M methanolic hydrogen chloride (75 ml) at room temperature for 48 h, after which the insoluble polysaccharide was removed and shaken with fresh 0.1 M methanolic hydrogen chloride (75 ml) for a further 48 h. The polysaccharide was isolated as above (0.62 g; 81%) [Found: SO_4^{2-}, 15.5%]. Paper chromatography of an acid hydrolysate of this material revealed the presence of galactose and xylose.

In a fourth experiment, polysaccharide (1.0 g) was shaken with 0.15 M methanolic hydrogen chloride (75 ml) for 72 h. The insoluble polysaccharide was removed and isolated as before (0.69 g; 87%) [Found: SO_4^{2-}, 18.8%].

The partially desulphated products from the third and fourth experiments (0.62 g; SO_4^{2-}, 15.5%; and 0.69 g; SO_4^{2-}, 18.8%) were combined, shaken with 0.15 M methanolic hydrogen chloride (90 ml) for 48 h at room temperature and isolated as before (0.64 g) [Found: SO_4^{2-}, 3.9%].

A large quantity of polysaccharide (13.0 g; dried in vacuo over P_2O_5 for 48 h) was then subjected to five treatments with 0.15 M methanolic hydrogen chloride (48 h each) at room temperature. The polysaccharide was isolated by dialysis and freeze-drying after each treatment. The final product (5 g) had a sulphate content of 1.2%. The infrared spectrum (KBr disc) of this desulphated polymer is given in Fig. 2.

2. 1. 11 Periodate oxidation of polysaccharides

Experiment A.

To polysaccharide (368.9 mg; dried at 60° over P_2O_5 in vacuo for 48 h) in water (50 ml) was added 0.06 M sodium metaperiodate (50 ml) and the mixture allowed to stand in the dark at room temperature. Aliquots (5 ml) were withdrawn at regular intervals and the reduction of periodate measured by the arsenite method[87] (Table IIa).

After 144 h, the solution remaining was treated with an excess of ethylene glycol. Sodium borohydride (0.4 g) was then added and the mixture allowed to stand overnight. The polysaccharide alcohol (194 mg) was isolated by freeze-drying after dialysis. Paper chromatographic examination of a neutralised, acid hydrolysate of the polysaccharide alcohol revealed galactose only (solvents A and B; spray 1). A portion of the polysaccharide alcohol (20 mg) was hydrolysed, reduced (sodium borohydride), acetylated[84] and examined by g.l.c. No peak corresponding to xylitol acetate was detected.

Experiment B.

Polysaccharide (27.63 mg) and desulphated polysaccharide

(SO_4^{2-}, 1.2%; 19.37 mg) were dissolved separately in water (5 ml each) and 0.02937 M sodium metaperiodate (5 ml) was added to each solution. The solutions were set aside at room temperature in the dark, and aliquots (0.10 ml) were withdrawn at intervals, diluted 250 times, and the reduction of periodate measured spectrophotometrically.[88] (Tables IIa and IIb.)

2.1.12 Methylation of desulphated polysaccharide

Polysaccharide (4.75 g; SO_4^{2-}, 1.2%) was dissolved in dimethyl sulphoxide (100 ml). Powdered sodium hydroxide (30 g) and dimethyl sulphate (15 ml) were added with stirring over a period of 5 h. The mixture was stirred for a further 16 h and sufficient concentrated ammonia was then added, followed by vigorous shaking, to decompose the dimethyl sulphate. After the addition of water (200 ml), the mixture was dialysed against distilled water for 5 days. The solution was concentrated to a small volume and the polysaccharide isolated by freeze-drying (5.0 g). The above methylation procedure was repeated and after a third methylation, the solution containing the partially methylated polymer was dialysed, concentrated, and extracted with chloroform (5 x 100 ml). Evaporation of the combined chloroform solutions yielded a gum (A; 1.0 g). The aqueous solution containing the chloroform-insoluble material, on concentration and freeze-drying, yielded a partially methylated polysaccharide (3.62 g). This fraction was remethylated and extracted with chloroform as before yielding a gum (B; 0.20 g). The chloroform-insoluble material (2.90 g) was not further investigated.

Fractions A and B were combined (1.20 g) [Found: OMe, 36.0%] and dissolved in methyl iodide (25 ml). Silver oxide (8 g) was added in

small portions and the mixture gently refluxed for 8 h with stirring.[89] After filtration, the silver salts were thoroughly washed with chloroform and the combined filtrate and washings concentrated to yield the polysaccharide (0.93 g). Two further treatments with Purdie's reagents[89] afforded a product (0.94 g) [Found: OMe, 40.3%] (Theoretical for galactose: xylose, 6:1; OMe, 44.5%). The infrared spectrum of the product (in dry $CHCl_3$) showed a very small hydroxyl peak. Further treatments with Purdie's reagents failed to increase the methoxyl content.

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

The methylated, desulphated polysaccharide (OMe, 40.3%; 0.94 g) was dissolved in 90% aqueous formic acid[90] (30ml), and the solution was kept at 100° for 1 h. The solution was cooled and concentrated to a syrup under reduced pressure. The syrup was then hydrolysed (100° for 16 h) with 0.5 N sulphuric acid (30 ml). The hydrolysate was neutralised (barium carbonate), centrifuged, and concentrated to a syrup (0.70 g), which was applied to a charcoal-Celite column (1:1; 32 x 4 cm). The methylated sugars were eluted by applying a linear gradient of 0-3% methyl ethyl ketone in water over a volume of 8 l. Fractions (ca 25 ml) were collected, analysed by paper chromatography, and combined into the following nine fractions.

Fraction I. The syrup (149 mg) was shown by paper chromatography (solvents A and B) to be a mixture of mono-O-methyl- and di-O-methyl-galactoses. The syrup was separated into two fractions (Whatman No. 1 paper; solvent B; 6 h), and the fractions were examined as follows:

Mono-O-methyl fraction. The syrup (35 mg) was fractionated by paper chromatography (solvent B) into 1 (a) a syrup (7 mg) chromatographically (solvents A and B) identical with 4-O-methylgalactose, $[\alpha]_D^{20}$ - 3° (c 0.70); 1 (b) a syrup (5 mg) which on reduction (sodium borohydride), acetylation,[84] and g.l.c. examination of the resultant glycitol acetates revealed peaks with retention times identical to those of 6-O-methyl-, 2-O-methyl- and 4-O-methylgalactitol acetates, in the approximate molar ratio 2:2:1; and 1 (c) a chromatographically pure syrup (21 mg), which crystallised from methanol-ethyl acetate, and had m.p. 152-153° undepressed on admixture with authentic 2-O-methyl-D-galactose, $[\alpha]_D^{20}$ + 14° (4 min) → + 62° (c 0.42); lit.[91] m.p. 148-149°, $[\alpha]_D^{16}$ + 84.9° (final) (c 0.53).

Di-O-methyl fraction. The syrup (111 mg) was shown by paper chromatography (solvent B; spray 1) to contain 2,6-di-O-methylgalactose (R_{Gal} 6.0; red; major component), 2,4-di-O-methylgalactose (R_{Gal} 4.8; yellow, goes red on standing), and 2,3-di-O-methylgalactose (R_{Gal} 5.6; yellow, goes red on standing). A portion of the syrup (13 mg) was reduced with borohydride and acetylated.[84] G.l.c. examination of the glycitol acetates revealed the presence of peaks with retention times corresponding to those of 2,6-di-O-methylgalactose (T_E 3.94; major component), 2,4-di-O-methylgalactose (T_E 6.26), 4,6-di-O-methylgalactose (T_E 4.27; trace) and 2,3-di-O-methylgalactose (T_E 5.12). Paper chromatographic separation of the syrup (98 mg) (solvent B; 12 h) yielded chromatographically pure samples of 2,6-di-O-methylgalactose (10 mg), $[\alpha]_D^{20}$ + 56° (c 0.50) and 2,4-di-O-methylgalactose (16 mg), $[\alpha]_D^{20}$ + 30° (c 0.53). Andrews et al[92] reported $[\alpha]_D$ + 84° (c 0.4) for 2,6-di-O-methyl-D-galactose and $[\alpha]_D$ + 85° (c 0.3) for 2,4-di-O-methyl-D-galactose.

Fraction II. The syrup (63 mg) was shown (paper chromatography; solvent B; spray 1) to contain 2, 3-di-O-methylxylose (R_{TMG} 0.93; pink), 2, 4-di-O-methylxylose (R_{TMG} 0.86; pink), 2, 3, 4-tri-O-methylgalactose (R_{TMG} 0.68; brown) and a methylated uronic acid (trace; R_{TMG} 0.0, 0.43 (solvent A); pink). Separation of the syrup on Whatman No. 1 paper (solvent B; 6 h) followed by extraction of the appropriate portions of the papers with 50% aqueous methanol yielded the three major components, which were characterised by g. l. c. examination of their methyl glycosides. 2, 3, 4-Tri-O-methylgalactose (11 mg), $[\alpha]_D^{20}$ + 11° (c 0.55), gave a peak (T 7.41) identical to that obtained from authentic methyl 2, 3, 4-tri-O-methylgalactosides; 2, 4-di-O-methyl-D-xylose (8 mg), $[\alpha]_D^{20}$ + 21° (c 0.57), gave peaks (T 1.46, 1.92) identical to those obtained from authentic methyl 2, 4-di-O-methylxylosides; and 2, 3-di-O-methyl-D-xylose (8 mg), $[\alpha]_D^{20}$ + 20° (c 0.60) gave peaks (T 1.47, 1.74) identical to those given by authentic 2, 3-di-O-methylxylosides, as well as a peak (T 1.30) considered to have arisen from the presence of a small amount of methyl 3, 4-di-O-methylxylosides. In support of this, the 2, 3-di-O-methylxylose was revealed, to a small extent, with triphenyltetrazolium hydroxide, while demethylation[76] of a portion yielded xylose as the only monosaccharide (paper chromatography).

Fraction III. The syrup (49 mg), which was shown by paper chromatography (solvent B; spray 1) to be 2, 4, 6-tri-O-methylgalactose (R_{TMG} 0.73; red-brown) together with a trace of the 2, 3, 4-isomer, crystallised from ether-ligroin. The crystals had $[\alpha]_D^{16}$ + 120° (4 min) → + 89° (c 0.63), m. p. and mixed m. p. 102-103° with authentic 2, 4, 6-tri-O-methyl-D-galactose,[93] m. p. 104-106°, $[\alpha]_D^{20}$ + 96° (c 1.4). The sugar (25 mg), freshly distilled aniline (65 mg), and a trace of glacial

acetic acid were heated with ethanol (2 ml) under reflux for 4 h. The "anilide" crystallised on cooling and, after recrystallisation from ethanol, had m. p. and mixed m. p. 173-174° with authentic 2, 4, 6-tri-O-methyl-N-phenyl-D-galactosylamine. Clingman and Nunn[94] reported m. p. 170. 5-171. 5°.

Fraction IV. This syrup (145 mg) was shown by paper chromatography (solvent B) to be a mixture of 2, 3, 6- and 2, 4, 6-tri-O-methylgalactose in the approximate ratio of 2:1.

Fraction V. The syrup (80 mg), chromatographically identical with 2, 3, 6-tri-O-methylgalactose (R_{TMG} 0. 83, solvent B; red-brown, spray 1) had $[\alpha]_D^{16}$ - 56° (c 0. 50). Nunn and Parolis[91] reported $[\alpha]_D^{18}$ + 90° (c 0. 37) for 2, 3, 6-tri-O-methyl-D-galactose. The syrup (35 mg) was oxidised with bromine water (7 ml; 96 h) and, after removal of bromine by aeration, the solution was neutralised with silver carbonate and filtered, and the silver ions precipitated with hydrogen sulphide. The filtrate from this treatment was evaporated to dryness in vacuo, and the residue extracted with dry ether. Colourless needles were obtained on concentration of the extract which, after recrystallisation from ether, had m. p. 97-99°. The m. p. was depressed on admixture with authentic 2, 3, 6-tri-O-methyl-D-γ-galactonolactone m. p. 97-99°;[91] the infrared spectrum (KBr disc) was identical to that of the authentic D-compound.

Fraction VI. The syrup (64 mg) was shown (paper chromatography) to be a mixture of 2, 3, 6-tri-O-methylgalactose and a methylated uronic acid (R_{TMG} 0. 53, solvent A; pink, spray 1). The sugar mixture was separated on Whatman No. 1 paper (solvent B; 6 h). Extraction of the appropriate portions of the paper with 50% aqueous methanol, followed by concentration, afforded a chromatographically pure syrup (23 mg),

R_{TMG} 0.53 (solvent A). The syrup was shaken with Amberlite IR-120 (H^+) resin to yield the free acid, $[\alpha]_D^{17}$ + 45° (c 0.40); lit.[95] $[\alpha]_D$ + 58° for 2, 3, 4-tri-O-methyl-D-glucuronic acid. The free acid was converted into the methyl ester methyl glycoside by refluxing with 4% methanolic hydrogen chloride for 7 h. The cooled solution, after neutralisation with silver carbonate, was filtered and concentrated to a syrup. G.l.c. examination of the methyl ester methyl glycoside showed peaks having retention times identical with those of the methyl ester methyl glycoside of authentic 2, 3, 4-tri-O-methylglucuronic acid (T 2.50, 3.24). Reduction of the carboxyl group[96] was achieved by allowing a 2% solution of the methyl ester methyl glycoside to stand with an equal weight of sodium borohydride for 16 h. The solution was then shaken with Amberlite IR-120 (H^+) resin, filtered, and evaporated to dryness. The borate was removed by repeated distillation with methanol. G.l.c. examination of the methyl glycosides showed peaks having retention times identical with those of authentic methyl 2, 3, 4-tri-O-methylglucosides (T 2.57, 3.69). Acid hydrolysis of the methyl glycosides (N sulphuric acid; 100°; 3 h) yielded a syrup, chromatographically identical (solvent B; spray 1) with 2, 3, 4-tri-O-methylglucose, which on demethylation[76] gave rise to glucose. Thus the methylated uronic acid is 2, 3, 4-tri-O-methyl-D-glucuronic acid.

Fraction VII. The syrup (42 mg), which contained 2, 3, 4, 6-tetra-O-methylgalactose (R_{TMG} 1.0, solvent B; red-brown, spray 1) and the methylated uronic acid present in Fraction VI, was separated on Whatman No. 1 paper (solvent B; 6 h) to yield chromatographically pure 2, 3, 4, 6-tetra-O-methylgalactose (30 mg), $[\alpha]_D^{17}$ 0° (c 0.50). The aniline derivative, after several recrystallisations from ethanol, had m.p. 178-180°, which could not be increased by further recrystallisations, $[\alpha]_D^{20}$ 0° (c 0.49 in acetone). The mixed m.p. (with authentic 2, 3, 4, 6-tetra-O-methyl-N-

phenyl-D-galactosylamine,[97] m. p. 192-194°) was 178-190°. The infrared spectrum (KBr disc) was identical with that of the "anilide" of 2, 3, 4, 6-tetra-O-methyl-D-galactose. Bell and Baldwin[98] reported m. p. 179-180°, $[\alpha]_D$ 0° (c 2. 5 in acetone) for the "anilide" of 2, 3, 4, 6-tetra-O-methyl-DL-galactose.

Fraction VIII. The syrup (36 mg) was shown by paper chromatography (solvents A and B) to be a mixture of 2, 3, 4-tri-O-methylxylose and 2, 3, 4, 6-tetra-O-methylgalactose.

Fraction IX. The syrup (50 mg), chromatographically identical to 2, 3, 4-tri-O-methylxylose (R_{TMG} 1. 11, solvent B; pink, spray 1), was crystallised from ether-ligroin, m. p. and mixed m. p. 89-90°, $[\alpha]_D^{19}$ + 48° (4 min) → + 19° (c 0. 50); lit.[60] m. p. 89-90°, $[\alpha]_D^{15}$ + 20. 3° (c 1. 1). The sugar(45 mg), dry pyridine (3 ml) and p-nitrobenzoyl chloride (200 mg) were heated at 90° for 2 h and then left overnight at room temperature. A saturated aqueous solution of sodium bicarbonate was added dropwise to the reaction mixture until no further effervescence occurred. Water (5 ml) was added and the product extracted with chloroform (3 x 15 ml). The extract was dried (magnesium sulphate) and evaporated to a syrup which crystallised on trituration with ethanol. The p-nitrobenzoate, after recrystallisation from ethanol, had m. p. 133-134°; lit.[59] m. p. 135°.

2. 1. 14. Partial hydrolysis of polysaccharide

Polysaccharide (1. 0g) in N sulphuric acid (20 ml) was heated by immersion of the flask in a boiling-water bath. Samples (1 ml) were removed at regular intervals, neutralised ($BaCO_3$), concentrated and

examined on paper chromatograms run in solvent A. The chromatograms were characterised by streaking but it appeared that 2 - 2.5 h under the above conditions gave the maximum concentration of oligosaccharides. A large quantity of polysaccharide (18.0 g) was then hydrolysed similarly for 2 h and the hydrolysate, after neutralisation ($BaCO_3$) and centrifugation, was deionised (Amberlite IR-120 (H^+) and IRA-400 (acetate) resins). The aqueous eluate containing the neutral fragments was concentrated to a syrup (3.82 g). The acid components were eluted from the IRA-400 (acetate) column with N sulphuric acid (4.0 l). The eluate was neutralised with barium carbonate, concentrated to about 200 ml, and passed through a column of Amberlite IR-120 (H^+) resin. After removal of the acetic acid from the latter eluate by freeze-drying, the product was dissolved in water (50 ml) and neutralised with ammonia. The ammonium salts were isolated by freeze-drying (13.0 g).

2.1.15 Separation and characterisation of the neutral components of the partial hydrolysate

The neutral syrup (3.82 g) in the minimum quantity of water, was 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 30 ml) were subsequently combined on the evidence of paper chromatographic examination into the following ten fractions.

Fraction I. The syrup (2.69 g), eluted with water (10 l), contained (paper chromatography using solvents A and B) galactose (major), xylose, and a minute trace of glucose.

Fraction II. The syrup (0.025 g) eluted with 2% aqueous ethanol (1.5 l), showed the presence of galactose and a substance with R_{Gal} 2.1 (solvent B). The syrup was separated on Whatman No. 1 paper, using solvent A. Extraction of the appropriate portions of the paper with 50% aqueous methanol, followed by concentration afforded a chromatographically pure product (9 mg), having R_{Gal} 2.1 (solvent B), $[\alpha]_D^{21}$ + 28° (c 0.5). The sugar moved with the mobility of authentic 3-O-methylgalactose in solvent systems A, B, and C, and gave spots of the same colour as given by this sugar with spray 1. Demethylation[76] of the sugar (2 mg) with 48% hydrobromic acid (0.2 ml) on a boiling-water bath for 5 min, followed by paper chromatography (solvents A and B; spray 1), revealed the presence of galactose and unchanged material. G.l.c. examination of the glycitol acetate,[84] prepared from the sugar (3 mg), gave a peak having the same retention time as the glycitol acetate of authentic 3-O-methylgalactose. The low value obtained for the optical rotation (cf 3-O-methyl-D-galactose $[\alpha]_D$ + 109° [99]) is possibly due to the sugar being a mixture of 3-O-methyl-D- and 3-O-methyl-L-galactose.

Fraction III. The syrup (0.531 g), eluted with 5% aqueous ethanol (6.5 l), was shown by paper chromatography to be a mixture of three oligosaccharides having R_{Gal} 0.24 (major), 0.31, and 0.17 (trace) (solvent B). An aqueous solution of the syrup was decolourised with charcoal, filtered and evaporated to a syrup. Crystallisation from 80% aqueous ethanol gave colourless needles (185 mg), which, after recrystallisation from the same solvent, had m.p. 237-238° (d), $[\alpha]_D^{19}$ - 48° (c 0.5) (final), R_{Gal} 0.24 (solvent B), 0.34 (solvent A). Paper chromatography (solvent A) of a partial acid hydrolysate (2 mg) revealed the presence of galactose and the original material.

A portion (40 mg) was reduced with sodium borohydride (40 mg) in water (5 ml) for 24 h, and then shaken with Amberlite resin (IR-120 (H^+) form). After filtering off the resin, the solution was evaporated to dryness, and the borate removed by repeated distillation with methanol. Acid hydrolysis of the non-reducing syrup, followed by separation of the resultant mixture on Whatman No. 1 paper, using solvent A, afforded two fractions. The first fraction was recrystallised from methanol (charcoal) giving galactitol, m. p. and mixed m. p. 186-187°. The second fraction was recrystallised from methanol (charcoal) giving D-galactose, $[\alpha]_D^{17}$ + 117° (5 min) → + 78° (final) (c 0.56).

Another portion of the oligosaccharide (20 mg) was hydrolysed (N sulphuric acid for 3 h at 100°), neutralised ($BaCO_3$), centrifuged and evaporated to dryness. The residue was crystallised from methanol and, after recrystallisation from the same solvent, gave galactose, m. p. 160-161°, $[\alpha]_D^{18}$ 0° (c 0.57). A further portion of the oligosaccharide (10 mg) in redistilled N, N-dimethylformamide (1.0 ml) was cooled to 0° and redistilled methyl iodide (1.0 ml) and dry silver oxide (1.0 g) added.[100] The mixture was stirred in the dark at 0° for 3 h and then for 45 h at room temperature. The product was filtered and the silver salts thoroughly washed with chloroform. The filtrate and washings were concentrated to dryness and traces of N, N-dimethylformamide removed under high vacuum (0.1 torr; 40°; 5 min). The dry residue in chloroform, was filtered, and concentrated to a syrup (9.2 mg), which was given one treatment with Purdie's reagents.[89] T. l. c. (spray 4) of the product indicated that methylation was complete. A portion of the methylated product on hydrolysis (N sulphuric acid) and examination by t. l. c. (spray 4) revealed spots having the mobilities of 2, 3, 4, 6-tetra-O-methylgalactose (blue-grey; R_{TMG} 1.0) and 2, 3, 6-tri-O-methylgalactose

(brown-grey; R_{TMG} 0.88). The remainder of the methylated product was refluxed with 3% methanolic hydrogen chloride for 6 h and the derived methylglycosides examined by g.l.c. Peaks corresponding to 2,3,4,6-tetra-O-methylgalactose (T 1.88) and 2,3,6-tri-O-methylgalactose (T 3.29, 4.02, 4.26, and 4.58), in the molar ratio 1.0: 1.02, were observed. The above evidence indicates that this disaccharide is 4-O-β-D-galactopyranosyl-L-galactose(1). The β-configuration is assumed from the specific rotation. The α-linked disaccharide would be expected to have a positive specific rotation.

The mother liquor from Fraction III, after the crystallisation of 4-O-β-D-galactopyranosyl-L-galactose, was separated on Whatman No. 1 paper, using solvent B. This afforded a chromatographically pure syrup (25 mg) having R_{Gal} 0.31 (solvent B), 0.34 (solvent A), $[\alpha]_D^{19}$ -20° (c 0.6). Paper chromatography of a partial, acid hydrolysate revealed the presence of galactose and the original material. Complete hydrolysis of an aliquot (9 mg) gave galactose having $[\alpha]_D^{18}$ 0° (c 0.43). Reduction of the reducing moeity of another aliquot (12 mg) followed by hydrolysis and separation of the products on Whatman No. 1 paper, using solvent A, gave chromatographically pure galactose, $[\alpha]_D^{21}$ - 73° (c 0.48). Methylation of a further aliquot (5.7 mg) as above gave, on g.l.c. examination of a methanolysate, peaks with the retention times of methyl 2,3,4,6-tetra-O-methylgalactosides (T 1.80) and methyl 2,4,6-tri-O-methylgalactosides (T 4.05 and 4.50), in the molar ratio 1.0:1.05. The presence of these sugars was confirmed by paper chromatography of an acid hydrolysate of a portion of the fully methylated oligosaccharide. These results indicate that this disaccharide is 3-O-α-L-galacto-pyranosyl-D-galactose (2). The α-configuration is assumed from the

negative specific rotation. The β-linked disaccharide would be expected to have a positive specific rotation.

Fraction IV. The syrup (0.108 g), eluted with 5% aqueous ethanol (11 l), was shown by paper chromatography to be a mixture of oligosaccharides, R_{Gal} 0.36 (major), 0.34, 0.26, 0.17, and 0.13 (the last three in trace quantities) (solvent A). Separation of this fraction on Whatman No. 1 paper, using solvent A, yielded a chromatographically pure syrup (41 mg) having R_{Gal} 0.36 (solvent A), which readily crystallised from methanol (charcoal) and had m.p. and mixed m.p. 203-205° with 4-O- β -D-galactopyranosyl-D-galactose;[97] $[\alpha]_D^{17}$ + 84° (3 min) → + 70° (final) (c 0.5). The infrared spectrum (KBr disc) was identical with that of 4-O- β -D-galactopyranosyl-D-galactose (3). A portion of the disaccharide was methylated and methanolysed, and the derived methyl glycosides examined by g.l.c. Peaks corresponding to 2, 3, 4, 6-tetra-O-methylgalactose (T 1.78) and 2, 3, 6-tri-O-methyl-galactose (T 3.14, 3.78, 4.14, and 4.45), in the molar ratio of 1.0: 1.06, were observed.

Fraction V. The syrup (0.018 g), eluted with 7.5% aqueous ethanol (8.5 l), was shown by paper chromatography (solvent A) to be a mixture of three oligosaccharides having R_{Gal} 0.13, 0.17, and 0.36. The fraction was not further examined.

Fraction VI. The syrup (0.206 g) eluted with 10% aqueous ethanol (15 l), contained four oligosaccharides R_{Gal} 0.1 (major), 0.32, 0.17 (trace), and 0.24 (trace) (solvent A). Separation on Whatman No. 1 paper (solvent A), followed by extraction of the appropriate portions of the papers with 50% aqueous methanol, afforded a chromatographically pure syrup (80 mg), R_{Gal} 0.1 (solvent A), which,

after recrystallisation from methanol, had m.p. 229-230° (d), $[\alpha]_D^{19}$ - 36° (3 min) → -44° (final) (c 0.50). Paper chromatography of a neutralised partial acid hydrolysate revealed the presence of 4-O- β -D-galactopyranosyl-L-galactose, 3-O- α -L-galactopyranosyl-D-galactose, galactose, and the original material. Complete acid hydrolysis gave galactose having $[\alpha]_D^{20}$ + 28° (c 0.49). The value for the specific rotation indicates a D:L-isomer ratio of 2:1. Reduction of the saccharide, followed by paper chromatography of a partial acid hydrolysate of the non-reducing syrup, revealed the presenceof 4-O- β -D-galactopyranosyl-L-galactose and galactose. A portion of the oligosaccharide (5 mg) was methylated and hydrolysed, and the products examined by paper chromatography (solvent B; spray 1). Spots with the mobilities of 2, 3, 4, 6-tetra-O-methylgalactose (R_{TMG} 1.0), 2, 4, 6-tri-O-methylgalactose (R_{TMG} 0.73) and 2, 3, 6-tri-O-methylgalactose (R_{TMG} 0.83) were observed. These results indicate that this trisaccharide is O- β -D-galactopyranosyl (1 → 4)-O- α -L-galactopyranosyl (1→3)-D-galactose (4).

Fraction VII. The syrup (0.144 g), eluted with 10-15% aqueous ethanol (11.5 l), was shown (paper chromatography) to be a mixture of Fractions VI and VIII.

Fraction VIII. The syrup (0.053 g), eluted with 20% aqueous ethanol (4 l), contained an oligosaccharide having R_{Gal} 0.04, and traces of three other oligosaccharides R_{Gal} 0.01, 0.1, and 0.14 (solvent A). Separation of this fraction on Whatman No. 1 paper (solvent A) gave a chromatographically pure syrup (24 mg), R_{Gal} 0.04, $[\alpha]_D^{18}$ - 66° (c 0.58). Partial acid hydrolysis, followed by paper chromatography (solvents A and B), revealed the presence of galactose, 4-O- β -D-galactopyranosyl-L-galactose, 3-O- α -L-galactopyranosyl-D-galactose, O- β -D-galacto-

pyranosyl (1→4)-O- α -L-galactopyranosyl (1→3)-D-galactose, an oligosaccharide having R_{Gal} 0.13 (solvent A), and the starting material. Complete acid hydrolysis gave galactose having $[\alpha]_D^{22}$ $0^\circ \pm 3^\circ$ (c 0.35). Reduction of the oligosaccharide as above, followed by paper chromatography of the partial acid hydrolysate of the non-reducing syrup, revealed the presence of O- β -D-galactopyranosyl (1→4)-O- α -L-galactopyranosyl (1→3)-D-galactose, 4-O- β -D-galactopyranosyl-L-galactose, 3-O- α -L-galactopyranosyl-D-galactose, and galactose. A portion of the oligosaccharide was methylated and hydrolysed and paper chromatographic examination (solvents A and B; spray 1)of the products revealed the presence of 2, 3, 4, 6-tetra-O-methylgalactose, 2, 3, 6-tri-O-methylgalactose, and 2, 4, 6-tri-O-methylgalactose. These results indicate that this is a tetrasaccharide with a probable composition of O- β -D-galactopyranosyl (1→4)-O- α -L-galactopyranosyl (1→3)-O-D-galactopyranosyl (1→4)-L-galactose (5).

Fraction IX. The syrup (0.028 g), eluted with 20-25% aqueous ethanol (4.5 l), was a mixture of two oligosaccharides R_{Gal} 0.04 and 0.06 (trace) (solvent A).

Fraction X. The syrup (0.022 g), eluted with 25-40% aqueous ethanol (2.5 l), consisted mainly of an oligosaccharide having R_{Gal} 0.02, together with traces of two other oligosaccharides, R_{Gal} 0.04 and 0.06 (solvent A).

2. 1. 16 Separation and characterisation of the acidic components of the partial hydrolysate

An aqueous solution of the acidic sugars (10 g) in the ammonium form

was applied to a charcoal-Celite column (1:1; 27 x 4 cm) and eluted with water and then aqueous ethanol to give three main fractions. The first fraction (6.82 g), eluted with water (600 ml), consisted of inorganic material. The third fraction (1.65 g), eluted with aqueous ethanol (0 - 20%; 9 l), was shown by paper chromatography (solvents A and D) to contain high molecular weight acidic fragments. These fractions were not further investigated. The second fraction (0.58 g), eluted with water (2.1 l), was separated on Whatman No. 1 paper for 16 h, using solvent A. The appropriate portions of the papers were extracted with 50% aqueous methanol. The fractions, after the addition of ammonia to pH8, were concentrated to dryness and reseparated on Whatman No. 1 paper (solvent D; 16 h). The following chromatographically homogeneous compounds (solvents A and D) were obtained as their ammonium salts.

Acidic sugar I. The syrup (50 mg) had R_{Gal} 0.75 (solvent D; yellow, spray 1), $[\alpha]_D^{21}$ - 32° (c 0.56) calculated as the ammonium salt of a hexose monosulphate from the sugar concentration found by the method of Dubois et al.[86] A neutralised, acid hydrolysate (N sulphuric acid; 100°; 12 h) gave a single spot with the mobility of galactose (paper chromatography; solvents A and B). The sugar had a DP of 1.0 and a molar ratio of galactose[86] to sulphate of 1.0:1.13. A portion of the sugar (8.5 mg) in redistilled N,N-dimethylformamide (1.0 ml) was cooled to 0° and redistilled methyl iodide (1.0 ml) and dry silver oxide (1.0 g) added.[100] The mixture was stirred in the dark at 0° for 3 h and then for 21 h at room temperature. The mixture was filtered and the silver salts thoroughly washed with chloroform. The filtrate and washings were concentrated to dryness and traces of N,N-dimethylformamide removed under vacuum (0.1 torr; 40°; 5 min). The dry residue was then given one treatment with Purdie's reagents.[89] T.l.c. (spray 4)

of the product indicated that methylation was complete. The methylated product was refluxed with 3% methanolic hydrogen chloride for 6 h and g. l. c. examination of the derived methyl glycosides revealed peaks characteristic of methyl 2, 4, 6-tri-O-methylgalactosides (T 4. 05, 4. 50). Hydrolysis (N sulphuric acid) of a portion of the methylated sugar and examination of the product by paper chromatography (solvents A and B) revealed a single spot having the mobility of 2, 4, 6-tri-O-methyl galactose. The above evidence indicates that this sulphated monosaccharide is L-galactose 3-sulphate. Peat et al[13] reported $[\alpha]_D^{18}$ + 45° for authentic D-galactose 3- sulphate (barium form).

Acidic sugar II. The syrup(25 mg) had R_{Gal} 0. 56 (solvent D; orange-brown, spray 1), $[\alpha]_D^{20}$ - 43° (c 0. 56) calculated as the ammonium salt of a hexose monosulphate. Hydrolysis (N sulphuric acid; 100°; 12 h) followed by neutralisation (barium carbonate) and paper chromatography (solvents A and B) revealed a single spot with the mobility of galactose. The sugar had a DP of 1. 0 and a molar ratio of galactose[86] to sulphate of 1. 0:1. 18. A sample (6 mg) was methylated as above and the product methanolysed for 6 h. T. l. c. of the methylglycosides (spray 4) revealed two spots with mobilities identical to those given by authentic methyl 2, 3, 4-tri-O-methylgalactosides, while g. l. c. examination revealed a single peak (T 7. 38) corresponding to methyl 2, 3, 4-tri-O-methylgalactosides. These results indicate that this sulphated monosaccharide is L-galactose 6-sulphate. Turvey and Williams[7] reported $[\alpha]_D$ + 47° for authentic D-galactose 6-sulphate (sodium form).

Acidic sugar III. The syrup (69 mg) had R_{Gal} 0. 31 (solvent A; orange-brown, spray 1), $[\alpha]_D^{20}$ + 24° (c 0. 50; free acid form). The sugar gave a positive reaction with triphenyltetrazolium hydroxide and on

complete acid hydrolysis yielded galactose and glucuronic acid (paper chromatography; solvents A and B). The saccharide (36 mg) was shaken with Amberlite IR-120 (H^+) resin and then refluxed with 2% methanolic hydrogen chloride (10 ml) for 6 h. The solution was neutralised with silver carbonate, filtered, and concentrated to dryness yielding the methyl ester methyl glycoside (30 mg) (i). Part of (i) (27 mg) was treated with sodium borohydride (60 mg) in water (5 ml) for 16 h at room temperature. The solution was shaken with Amberlite IR-120 (H^+) resin, and boric acid was removed by repeated distillation with methanol. A portion (19 mg) of the product (ii) was hydrolysed (N sulphuric acid; 100°; 4 h) to yield galactose and glucose in the ratio 1.0: 1.08 (estimated by g.l.c. of the derived glycitol acetates[84]). The galactose/glucose mixture was separated on Whatman No. 1 paper (solvent B; 40 h) giving D-glucose (4 mg), $[\alpha]_D^{21}$ + 40° (c 0.40) and L-galactose (4 mg), $[\alpha]_D^{21}$ - 65° (c 0.40). The remainder of (ii) (2.5 mg) was methylated and the product methanolysed for 6 h. G.l.c. examination of the derived methyl glycosides showed peaks corresponding to methyl 2,3,4,6-tetra-O-methylglucosides (T 0.98, 1.45) and methyl 2,3,6-tri-O-methylgalactosides (T 3.30, 4.29, 4.68).

The remaining methyl ester methyl glycoside (i) (2 mg) was methylated and subjected to borohydride reduction. The product was methanolysed and on g.l.c. examination gave peaks corresponding to methyl 2,3,4-tri-O-methylglucosides (T 2.58, 3.69) and methyl 2,3,6-tri-O-methylgalactosides (T 3.17, 4.29, 4.60). The above evidence suggests that this aldobiuronic acid is 4-O-α-D-glucuronosyl-L-galactose. The anomeric configuration follows from the specific rotation; lit. $[\alpha]_D^{21}$ + 110° (c 2.1) for 4-O-α-D-glucuronosyl-D-galactose (Ba salt)[101] and $[\alpha]_D$ + 15° for 4-O-β-D-glucuronosyl-D-galactose.[102]

Acidic sugar IV. The syrup (30 mg), R_{Gal} 2.18 (solvent A), 0.20 (solvent D; orange, spray 1) was devoid of sulphate, had $[\alpha]_D^{20}$ + 51° (c 0.43) (ammonium form), and on total acid hydrolysis yielded galactose as the only reducing sugar. The sugar (0.6 mg) was refluxed with 3% methanolic hydrogen chloride for 3 h. After neutralisation with lead carbonate, the product was examined by g.l.c. on column (c) and a peak corresponding to that of methyl pyruvate dimethyl acetal was observed. A portion of the sugar (5 mg) was methylated, hydrolysed, and paper chromatographic examination of the product (solvents A and B) revealed the presence of 2,3-di-O-methylgalactose. The hydrolysate of the methylated sugar was reduced (sodium borohydride), acetylated,[84] and on g.l.c. examination showed a peak with retention time identical with that of the glycitol acetate prepared from authentic 2,3-di-O-methylgalactose (T_E 4.42, column (c)). These results indicate that this sugar is 4,6-O (1′-carboxyethylidene)-D-galactose.

Acidic sugar V. The syrup (43 mg) was devoid of sulphate and had R_{Gal} 0.67 (solvent A; yellow, spray 1), $[\alpha]_D^{20}$ - 37.4° (c 0.60) (ammonium form). Total acid hydrolysis (N sulphuric acid; 100°; 16 h), followed by paper chromatography (solvents A and B), revealed galactose as the only reducing sugar. Partial acid hydrolysis (N sulphuric acid; 100°; 10 min), followed by paper chromatography, revealed the presence of galactose, acidic sugar IV, 4-O-β-D-galactopyranosyl-L-galactose, and the original sugar. A portion of the sugar (0.9 mg) was methanolysed for 3 h and g.l.c. examination of the neutralised product (column (c)) showed a peak corresponding to that of methyl pyruvate dimethyl acetal. The sugar was methylated: a portion of the product was methanolysed; the remainder was hydrolysed and converted into the alditol acetates.[84]

G. l. c. examination showed peaks corresponding to methyl 2, 3, 6-tri-O-methylgalactosides (T 3.23, 4.02, 4.28, 4.70; column (a)) and 1, 4, 5, 6-tetra-O-acetyl-2, 3-di-O-methylgalactitol (T_E 4.42; column (c)). The presence of these sugars was confirmed by paper chromatography (solvents A and B) of an acid hydrolysate of a sample of the fully methylated oligosaccharide. These results indicate that this sugar is 4-O-β - [4, 6-O(1′-carboxyethylidene)-D-galactopyranosyl] -L-galactose.

Acidic sugar VI. The syrup (15 mg), $[\alpha]_D^{20}$ + 4° (c 0.50) (ammonium form), was chromatographically different from V having R_{Gal} 1.15 (solvent A), 0.12 (solvent D; yellow, spray 1). The sugar was devoid of sulphate and on total hydrolysis gave galactose as the only reducing sugar. Partial, acid hydrolysis, followed by paper chromatography (solvents A and B) revealed the presence of galactose, acidic sugar IV, an unidentified sugar having R_{Gal} 0.33 (solvent A), and the original material. Methanolysis of a portion of the saccharide, followed by g. l. c. examination of the products showed a peak corresponding to that of methyl pyruvate dimethyl acetal (column (c)). The sugar was methylated, hydrolysed and paper chromatographic examination (solvents A and B) of the hydrolysate revealed spots with the mobilities of 2, 3-di-O-methyl- and 2, 3, 6-tri-O-methylgalactose. Insufficient material remained for further investigations.

Acidic sugar VII. The syrup (3.3 mg) was chromatographically identical to glucuronic acid, R_{Gal} 0.92 (solvent A), 0.09 (solvent D; yellow, goes red, spray 1). An optically clear solution of the sugar could not be obtained. The sugar was shaken with Amberlite IR-120 (H^+) resin, methanolysed and the derived methyl ester methyl glycoside treated with sodium borohydride. A portion of the product was

hydrolysed (N sulphuric acid; 100°; 4 h) and paper chromatographic examination revealed glucose (solvents A and B). The remaining product was methylated and peaks corresponding to 2, 3, 4, 6-tetra-O-methylglucose (T 1.02, 1.46) were observed on g.l.c.

Two further acidic sugars were obtained: VIII (2 mg), R_{Gal} 1.80 (solvent A), 0.30 (solvent D; yellow, spray 1); and IX (3 mg), R_{Gal} 2.20 (solvent A), 0.32 (solvent D; yellow, spray 1). These sugars were devoid of sulphate and were unchanged by total acid hydrolysis (N sulphuric acid; 100°; 16 h).

2. 2 Chaetangium erinaceum

2. 2. 1 Isolation and purification of the xylan. (Carried out by Dr. H. Parolis)

Wet Chaetangium erinaceum (6 kg) was exhaustively extracted with hot water, the pH being maintained at 6 by the addition of acetic acid. The extract was centrifuged yielding a clear mucilage which was precipitated into ethanol (5 vol.). The crude polysaccharide was washed with ethanol and dried in vacuo at 60° to give an off-white product (440 g). [Found: N, 1.8%]. Paper chromatographic examination of an acid hydrolysate (N sulphuric acid; 16 h; 100°) revealed the presence of xylose, mannose and galactose in the approximate ratio 6:1:1 (solvents A, B and C; spray 1). Polysaccharide (400g), in solution in hot water, was centrifuged, and the clear supernatant solution treated with Fehling's solution. The "copper complex" (Fraction A) was collected by decantation, washed thoroughly with water, and decomposed by masceration in a blender for 1 min at 0° with ethanol containing 5%(v/v) hydrochloric acid. The residue was washed with ethanol, until the washings were chloride-free, and finally acetone and dried in vacuo (192 g). Paper chromatographic examination of an acid hydrolysate revealed the presence of xylose and small amounts of galactose and mannose. The xylan was purified by a second precipitation as its "copper complex" as above. Finally it was dissolved in water, the solution centrifuged, and the clear supernatant solution poured into ethanol. The recovered polysaccharide was washed with ether, and dried in vacuo at 45° (Yield 130 g) [Found: $[\alpha]_D^{20}$ - 112° (c 0.91)]. Paper chromatographic examination of a hydrolysate in solvents A, B and C revealed the presence of xylose only.

The supernatant solution (Fraction B), obtained after removal of the "copper complex", was neutralised with acetic acid and dialysed against

running tap water (5 days). The dialysate was concentrated to a small volume and passed through Amberlite IR-120 (H^+) resin. The acid eluate was exactly neutralised with sodium hydroxide solution, after which the solution was concentrated and precipitated into ethanol (5 vol.). The polysaccharide so obtained was collected by centrifugation, washed with ethanol and dried in vacuo, affording an off-white fibrous product (70g). Chromatographic examination (solvents A, B and C) revealed galactose, mannose and xylose in the approximate ratio 1:1:2 and traces of glucose, arabinose and a sugar having chromatographic mobility R_{Gal} 0.24 (solvent B; yellow-brown, spray 1).

All subsequent experiments were performed on the xylan (Fraction A). The infrared spectrum (KBr disc) of the xylan is given in Fig. 3.

2. 2. 2 Periodate oxidation of polysaccharide

Polysaccharide (478.6 mg) dried at 70° (0.1 torr for 24 h) was dissolved in 0.100 M sodium metaperiodate (100 ml) and set aside at room temperature in the dark. Aliquots (5 ml) were withdrawn at regular intervals and the reduction of periodate estimated titrimetrically.[87] (Table IV).

2. 2. 3 Methylation of polysaccharide

To polysaccharide (4.8 g) dissolved in dimethyl sulphoxide (500 ml) was added powdered sodium hydroxide (200 g) and dimethyl sulphate (175 ml) with stirring over a period of 8 h under nitrogen.[103] During

the first 2 h the reaction was carried out at 20°. After stirring for another 16 h, the mixture was heated on a boiling-water bath for 1.5 h to decompose the dimethyl sulphate. Water (200 ml) was added to dissolve the sodium hydroxide and the mixture was cooled to 5° and neutralised with 10 N sulphuric acid. The sodium sulphate which precipitated was filtered off and washed with chloroform. The aqueous filtrate was extracted with chloroform (2 l; in portions) and the combined chloroform extracts were dried (sodium sulphate) and evaporated to a brown syrup in vacuo. The partially methylated xylan was fractionated by applying it in chloroform to a silica gel column (60-120 mesh; 150g; 48 x 3 cm). Elution of the column with chloroform-methanol, the amount of the latter solvent being increased in stages, yielded four fractions. The properties of these fractions are compared in Table V.

To fraction B (3.62g; OMe, 34.9%) dissolved in methyl iodide (85 ml) and N,N-dimethylformamide (3 ml), was added dry silver oxide (30g; in portions). The mixture was gently refluxed for 8 h with stirring. After filtration, the silver salts were extracted with boiling chloroform. Concentration of the combined filtrate and extracts yielded the methylated polysaccharide (3.35g) [Found: OMe, 38.3; Theoretical: OMe, 38.75%]. The infrared spectrum (in dry $CHCl_3$) of the product showed no hydroxyl peak (Fig. 3). Further treatment with Purdie's reagents[89] failed to increase the methoxyl content.

2.2.4 Hydrolysis of methylated polysaccharide and separation of the products

The methylated polysaccharide [0.5g; $[\alpha]_D^{20}$ - 73°

(c 0.82 in chloroform)], externally cooled with iced water, was dissolved in 72% sulphuric acid[104] (5 ml) and the solution was kept at room temperature for 1 h. Water (40 ml) was then added, and the solution kept at 100° for 4 h. The acid hydrolysate was cooled and extracted with freshly distilled dichloromethane[105] (4 x 50 ml portions) to give fraction (i), and a further 4 x 50 ml portions to give fraction (ii). The aqueous phase was neutralised with barium carbonate. The solids were separated by centrifugation and were carefully washed with water. The centrifugate and washings were concentrated to a syrup under reduced pressure at a bath temperature of 35° (fraction (iii)). Fractions (i) and (ii) were shaken with solid sodium bicarbonate, dried (sodium sulphate) and allowed to evaporate to dryness at room temperature in a dust-free atmosphere. Fractions (i) (58 mg), (ii) (25 mg) and (iii) (411 mg) were applied to Whatman No. 1 paper (solvent B) and separated into the following components.

2, 3, 4-tri-O-methyl-D-xylose. The syrup (12 mg), R_{TMG} 1.11 (solvent B), had $[\alpha]_D^{20}$ + 9.6° (c 1.04); lit.[60] $[\alpha]_D^{15}$ + 20.3° (c 1.1). A portion (0.5 mg) was refluxed with 3% methanolic hydrogen chloride for 6 h, and the derived methyl glycosides were examined by g.l.c. Peaks corresponding to 2, 3, 4-tri-O-methyl-xylose (T 0.49, 0.58) were observed.

2, 3-di-O-methyl-D-xylose. The syrup (269 mg), R_{TMG} 0.93 (solvent B), $[\alpha]_D^{20}$ + 22° (c 0.50), was not revealed on spraying with triphenyltetrazolium hydroxide. Chanda *et al*[106] reported $[\alpha]_D^{15}$ + 23° (c 1.0) for authentic 2, 3-di-O-methyl-D-xylose. A dilute alcoholic solution of the sugar (1 part) when treated with aniline (7 parts) and a trace of glacial acetic acid, gave a crystalline "anilide" which, after recrystallisation from ethyl

acetate containing a little light petroleum, had m. p. and mixed m. p. 147-148° with authentic 2, 3-di-O-methyl-N-phenyl-D-xylosylamine, $[\alpha]_D^{20}$ + 180° (c 0. 56 in ethyl acetate); lit.[107] m. p. 146°, $[\alpha]_D^{19}$ + 185° (c 0. 76 in ethyl acetate).

2, 4-di-O-methyl-D-xylose. The syrup (59 mg), R_{TMG} 0. 86 (solvent B), had $[\alpha]_D^{20}$ + 22° (c 0. 60); lit.[108] $[\alpha]_D^{17}$ + 21. 5° (c 0. 5). The sugar was not revealed on spraying with triphenyltetrazolium hydroxide and the aniline derivative, after several recrystallisations, had m. p. 163-164°, $[\alpha]_D^{20}$ - 85° (c 0. 47 in dioxan). Barker et al[109] reported m. p. 170°, $[\alpha]_D^{20}$ - 82° (in dioxan) for authentic 2, 4-di-O-methyl-N-phenyl-D-xylosylamine.

Mono-O-methylxyloses. The syrup (20 mg) was shown (paper chromatography; solvent B) to contain two components, R_{Xyl} 2. 32 and 2. 57. The mono-O-methylxyloses were not further investigated.

The hydrolysate of the methylated polysaccharide was devoid of unmethylated xylose.

2. 2. 5 Quantitative estimation of the relative molar percent O-methylxyloses from the methylated polysaccharide

The methylated polysaccharide (10 mg) was hydrolysed in a sealed tube using the sulphuric acid method of Garegg and Lindberg.[104] The hydrolysate was diluted with water and shaken with Amberlite IRA-400 (acetate) resin. The more volatile components were separated by extraction with dichloromethane. The combined dichloromethane extracts were shaken with solid sodium bicarbonate, dried (magnesium sulphate), filtered and allowed to evaporate to dryness at room temperature in a

dust-free atmosphere (fraction a). The aqueous phase was concentrated to a syrup under reduced pressure at a bath-temperature of 30° (fraction b). Fractions a and b were combined (7.5 mg), dissolved in pyridine (12 drops), and treated with hydroxylamine hydrochloride (7.5 mg) at 90° for 1 h.[110] Acetic anhydride (40 drops) was then added and heating continued for another hour. The cooled solution was chromatographed directly using column (c), operated at 180° and a nitrogen flow rate of 50 ml/min. The molar percent of a given sugar was estimated from the peak area of its acetylated nitrile (measured by triangulation) compared with that of 2,4-di-O-methylxylose, taking into account the relative molar response of the components. [Found:- molar response 2,3,4-tri-: 2,4-di- : 2,3-di-O-methylxylose: mono-O-methylxyloses is 1.742: 1.0: 1.065: 0.443].

2.2.6 Partial hydrolysis of polysaccharide

In order to determine the optimum conditions for the production of low molecular weight oligosaccharides, the polysaccharide (0.5 g) was heated on a boiling-water bath with 0.1 N sulphuric acid (20 ml). Aliquots (1 ml) were withdrawn at regular intervals, neutralised with barium carbonate, and analysed by paper chromatography (solvents A and B; spray 1). Hydrolysis for 2.75 h under the above conditions was found to yield the maximum concentration of oligosaccharides. Thus, polysaccharide (10 g) in 0.1 N sulphuric acid (400 ml) was heated on a boiling-water bath for 2.75 h. The hydrolysate was neutralised (barium carbonate), centrifuged, and evaporated to a syrup. The syrup was dissolved in the minimum quantity of water and applied to a charcoal-

Celite column (5.4 x 60 cm). The xylose was eluted with water, and the oligosaccharides with aqueous ethanol of increasing strength. Fractions (ca 30 ml) were analysed by paper chromatography and combined into nine major fractions.

Fraction I. The syrup (1.84 g), eluted with water (2.7 l), crystallised from methanol and after recrystallisation first from methanol and then ethanol, had $[\alpha]_D^{19}$ + 88° (3 min) → +19° (c 0.51), m.p. and mixed m.p. 144-145° with authentic D-xylose. The derived O-dibenzylidene dimethyl acetal[82] had m.p. and mixed m.p. 210-211°, $[\alpha]_D^{15}$ - 9° (c 1.04 in chloroform).

Fraction II. The syrup (523 mg), eluted with 0-5% aqueous ethanol (4.7 l), was shown (paper chromatography) to be a mixture of xylose (major) and two oligosaccharides, R_{Xyl} 0.38 and 0.68 (trace) (solvent A). Separation of this mixture on Whatman No. 1 paper (solvent A; 19 h), followed by extraction of the appropriate portions of the papers, yielded a chromatographically pure syrup (1) (112 mg), R_{Xyl} 0.38 (solvent A), 0.33 (solvent B), M_X 0.26. The syrup failed to crystallise until seeded with authentic xylobiose. It crystallised from aqueous ethanol-ethyl acetate as needles, $[\alpha]_D^{20}$ - 27° (c 0.52), m.p. and mixed m.p. 190-191°. Howard[67] reported $[\alpha]_D$ - 25.8°, m.p. 190° for xylobiose. Complete acid hydrolysis (N sulphuric acid; 100°; 2 h) yielded only xylose, and chromatograms of partial acid hydrolysates (0.1 N sulphuric acid; 100°; 15 min) showed no sugars other than the starting material and xylose. The saccharide (6 mg) in N,N-dimethylformamide (0.6 ml) was cooled to 0° and methyl iodide (1.0 ml) and dry silver oxide (0.6 g) added.[100] The mixture was stirred in the dark at 0° for 3 h and then for 21 h at room temperature

to give a partially methylated product which, after one treatment with Purdie's reagents,[89] was found to be completely methylated (t. l. c., spray 4). A portion of the methylated product was refluxed with 3% methanolic hydrogen chloride for 6 h, and the derived methyl glycosides were examined by g. l. c. Peaks corresponding to 2, 3, 4-tri-O-methylxylose (T 0. 49, 0. 62) and 2, 3-di-O-methylxylose (T 1. 54, 1. 78) were observed. The remainder of the methylated product was hydrolysed with 0. 1 N sulphuric acid in a sealed tube for 5 h at 100°. The solution was diluted, shaken with Amberlite IRA-400 (acetate) resin and extracted with dichloromethane (3 x 8 ml). The dichloromethane extract was neutralised with solid sodium bicarbonate, dried (magnesium sulphate), filtered and evaporated to dryness at room temperature in a dust-free atmosphere. The aqueous phase was concentrated to a syrup under reduced pressure at a bath-temperature of 30°. The dichloromethane extract was redissolved in a small quantity of dichloromethane and added to the syrup from the aqueous phase and the whole was allowed to evaporate to dryness at room temperature in a dust-free atmosphere. The acetylated nitrile derivatives were prepared[110] and examined by g. l. c. Peaks corresponding to 2, 3, 4-tri-O-methylxylose (T_X 0. 63) and 2, 3-di-O-methylxylose (T_X 1. 43), in the molar ratio 1. 0 : 0. 9, were observed.

Fraction III. The syrup (13. 5 mg), eluted with 5-7. 5% aqueous ethanol (2. 2 l), was a mixture of xylose (trace) and two oligosaccharides, R_{Xyl} 0. 68 and 0. 38 (trace) (solvent A). The main component (10 mg), after separation of the mixture on Whatman No. 1 paper (solvent A; 16 h), had R_{Xyl} 0. 68 (solvent A), 0. 78 (solvent B), M_X 0. 47, $[\alpha]_D^{20}$ - 17° (c 0. 75). Complete acid hydrolysis yielded only xylose, and partial hydrolysis, no sugars other than xylose and the starting material. The

sugar (4 mg) was methylated as before and a portion of the methylated product was methanolysed, and the derived methyl glycosides examined by g. l. c. Peaks corresponding to 2, 3, 4-tri-O-methylxylose (T 0. 48, 0. 61) and 2, 4-di-O-methylxylose (T 1. 53, 1. 97) were observed. G. l. c. examination of the acetylated nitrile derivatives prepared[110] from a hydrolysed sample of the methylated oligosaccharide, yielded peaks corresponding to 2, 3, 4-tri-O-methylxylose (T_X 0. 64) and 2, 4-di-O-methylxylose (T_X 1. 00), in the molar ratio 1. 0: 1. 1. This disaccharide is thus 3-O- β -D-xylopyranosyl-D-xylose (rhodymenabiose) (2); lit.[67] $[\alpha]_D^{22}$ - 18. 4° ± 0. 6° (c 3. 25).

Fraction IV. The syrup (450 mg), eluted with 7. 5-10% aqueous ethanol (15 l), was shown by paper chromatography (solvents A and B) to contain xylose (trace) and two oligosaccharides, R_{Xyl} 0. 13 and 0. 38 (trace) (solvent A). The major component (3), R_{Xyl} 0. 13 (solvent A), 0. 09 (solvent B), M_X 0. 22, crystallised readily from aqueous ethanol-ethyl acetate and, after recrystallisation from 85% aqueous ethanol, had $[\alpha]_D^{20}$ - 51° (c 0. 45), m. p. 216-217° alone and on admixture with authentic xylotriose; lit.[111] $[\alpha]_D^{25}$ - 47°, m. p. 205-206°. Paper chromatography of a partial, acid hydrolysate revealed the presence of xylose, xylobiose and the original material, and complete hydrolysis gave xylose. G. l. c. examination of the methanolysed methylated oligosaccharide showed peaks corresponding to 2, 3, 4-tri-(T 0. 48, 0. 60) and 2, 3-di-O-methylxylose (T 1. 50, 1. 76). A portion of the methylated oligosaccharide was hydrolysed and the derived monosaccharides were converted into their acetylated nitriles.[110] G. l. c. analysis of the acetylated nitriles showed peaks corresponding to 2, 3, 4-tri-(T_X 0. 63) and 2, 3-di-O-methylxylose (T_X 1. 43) in the molar ratio 0. 9: 2. 2.

Fraction V. The syrup (37 mg), eluted with 10-15% aqueous ethanol (4.5 l), was shown by chromatography in solvent A to contain a sugar having R_{Xyl} 0.23, and traces of three other sugars, R_{Xyl} 0.38, 0.13 and 0.05. Separation on Whatman No. 1 paper (solvent A; 65 h), followed by extraction of the appropriate portions of the paper with 50% aqueous methanol, afforded a syrup (30 mg), which although chromatographically pure in solvent A, was shown to be a mixture by electrophoresis and chromatography in solvent B : M_X 0.17 and 0.35, R_{Xyl} 0.20 and 0.23. The syrup was thus further fractionated by paper chromatography (solvent B; 5 days) into V (a) a syrup (8 mg), $[\alpha]_D^{20}$ - 38° (c 0.42), R_{Xyl} 0.20 (solvent B; pink, spray 1), M_X 0.17 ; and V (b) a syrup (11 mg), $[\alpha]_D^{20}$ - 37° (c 0.50), R_{Xyl} 0.23 (solvent B; orange-pink, spray 1), M_X 0.35. Paper chromatography of a partial, acid hydrolysate of fraction V (a) revealed the presence of xylose, rhodymenabiose, xylobiose and the original material. The sugar (1 mg) was dissolved in water (1 ml), and sodium borohydride (2 mg) added and the mixture allowed to stand for 16 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 A), revealed the presence of xylose and rhodymenabiose. A portion after methylation and methanolysis gave on g.l.c. analysis peaks with the same retention times as those of methyl 2,3,4-tri-O-methylxylosides (T 0.48, 0.58), methyl 2,4-di-O-methylxylosides (T 1.55, 2.03) and methyl 2,3-di-O-methylxylosides (T 1.55, 1.74). A further portion, after methylation and hydrolysis, was converted into the acetylated nitriles[110] which on examination by g.l.c. showed peaks corresponding to 2,3,4-tri-O-methylxylose (T_X 0.64),

2,4-di-O-methylxylose (T_X 1.00) and 2,3-di-O-methylxylose (T_X 1.41), in the molar ratio 1.1 : 1.0 : 0.9. The above evidence suggests that fraction V (a) is O- β -D-xylopyranosyl (1→3)-O- β -D-xylopyranosyl (1→4)-D-xylose (4).

Partial hydrolysis of a portion of fraction V (b) gave xylose, rhodymenabiose, xylobiose and the original material (paper chromatography). Reduction followed by hydrolysis and paper chromatography, showed, as the only reducing sugars, xylose and xylobiose. Methylation followed by methanolysis and g.l.c. of the methyl glycosides, showed the presence of components with the same retention times as methyl 2,3,4-tri-O-methyl-(T 0.48, 0.60), 2,4-di-O-methyl-(T 1.53, 1.99) and 2,3-di-O-methylxylosides (T 1.53, 1.77). G.l.c. examination of the acetylated nitrile derivatives,[110] prepared from a hydrolysed sample of the methylated oligosaccharide, yielded peaks corresponding to 2,3,4-tri-O-methylxylose (T_X 0.62), 2,4-di-O-methylxylose (T_X 1.00) and 2,3-di-O-methylxylose (T_X 1.41), in the molar ratio 0.9:1.1:1.0. These results indicate that this oligosaccharide is O-β -D-xylopyranosyl (1→4)-O- β -D-xylopyranosyl (1→3)-D-xylose(5).

Fraction VI. The syrup (514 mg), eluted with 15% aqueous ethanol (12 l), consisted mainly of two sugars R_{Xyl} 0.05 and 0.23, together with traces of three other sugars, R_{Xyl} 0.38, 0.13 and 0.08 (solvent A). Separation of a portion of this fraction (193 mg) on Whatman No. 1 paper in solvent A for 70 h, yielded a chromatographically pure syrup (6) (105 mg), R_{Xyl} 0.05 (solvent A), 0.02 (solvent B), M_X 0.19. The syrup crystallised from aqueous methanol-ethyl acetate and on recrystallisation from the same solvent had m.p. 214-215°, $[\alpha]_D^{20}$ -60° (c 0.55); lit.[111] m.p. 219-220°, $[\alpha]_D^{25}$ -60° for authentic xylotetraose. Partial hydrolysis

of a portion (0.1 N sulphuric acid; 100°; 20 min) gave xylose, xylobiose, xylotriose and the original material (paper chromatography; solvent A). Methylation followed by methanolysis and g.l.c. examination of the methyl glycosides, gave peaks with the same retention times as those of methyl 2,3,4-tri-O-methyl- (T 0.48, 0.61) and 2,3-di-O-methyl-xylosides (T 1.51, 1.76). Analysis of the acetylated nitriles,[110] prepared after methylation and hydrolysis of the saccharide, showed peaks corresponding to 2,3,4-tri-O-methylxylose (T_X 0.63) and 2,3-di-O-methylxylose (T_X 1.43), in the molar ratio 0.9:3.15.

Fraction VII. The syrup (335 mg), eluted with 15-16% aqueous ethanol (25.1 l), contained four oligosaccharides, R_{Xyl} 0.08 (major), 0.05, 0.13 and 0.23 (trace) (paper chromatography; solvent A). Separation of this fraction on Whatman No. 1 paper (solvent A; 90 h) gave a syrup (64 mg), DP 3.7, which although chromatographically pure, R_{Xyl} 0.08 (solvent A) and 0.06 (solvent B), was found to be a mixture by electrophoresis, M_X 0.33, 0.18. Partial hydrolysis of the syrup, followed by paper chromatography (solvents A and B), revealed the presence of xylose, rhodymenabiose, xylobiose, O-β-D-xylopyranosyl (1→3)-O-β-D-xylopyranosyl (1→4)-D-xylose, O-β-D-xylopyranosyl (1→4)-O-β-D-xylopyranosyl (1→3)-D-xylose, xylotriose and the original material. Reduction, followed by hydrolysis and paper chromatography (spray 1), revealed all the above sugars with the exception of the starting material. This suggests that the syrup is a mixture of O-β-D-xylopyranosyl (1→4)-O-β-D-xylopyranosyl (1→4)-O-β-D-xylopyranosyl (1→3)-D-xylose, O-β-D-xylopyranosyl (1→4)-O-β-D-xylopyranosyl (1→3)-O-β-D-xylopyranosyl (1→4)-D-xylose, O-β-D-xylopyranosyl (1→3)-O-β-D-xylopyranosyl (1→4)-O-β-D-xylopyranosyl (1→4)-O-β-D-

xylopyranosyl (1→4)-D-xylose, and/or O- β -D-xylopyranosyl (1→3)-O- β -D-xylopyranosyl (1→4)-O- β -D-xylopyranosyl (1→3)-D-xylose (7).

Fraction VIII. The syrup (323 mg), eluted with 16-20% aqueous ethanol (17.4 l), was shown (paper chromatography; solvent A) to contain four oligosaccharides, R_{Xyl} 0.015 (major), 0.08, 0.05 (trace) and 0.13 (trace). The syrup was dissolved in 85% aqueous methanol and crystallised on the addition of a few drops of ethyl acetate. The crystals (64 mg), after recrystallisation from 85% aqueous methanol-ethyl acetate, had R_{Xyl} 0.015 (solvent A), M_X 0.16, $[\alpha]_D^{20}$ -66° (c 0.48), m.p. 221-222°. Partial hydrolysis of this sugar, followed by paper chromatography, revealed the presence of xylose, xylobiose, xylotriose, xylotetraose and the original material. Methylation, followed by methanolysis and g.l.c. examination of the methyl glycosides showed the presence of components with the same retention times as methyl 2,3,4-tri-O-methyl- (T 0.48, 0.58) and 2,3-di-O-methylxylosides (T 1.52, 1.76). G.l.c. investigation of the acetylated nitriles,[110] prepared from a hydrolysed sample of the methylated oligosaccharide, showed peaks corresponding to 2,3,4-tri-O-methylxylose (T_X 0.63) and 2,3-di-O-methylxylose (T_X 1.43). These results indicate that this saccharide is xylopentaose (8). Whistler and Tu[111] reported $[\alpha]_D^{25}$ - 66° and m.p. 231-232° for authentic xylopentaose. $1/2H_2O$.

Fraction IX. The syrup (190 mg), eluted with 20-30% aqueous ethanol (21.6 l), consisted mainly of a saccharide, R_{Xyl} 0.03, together with traces of three other saccharides, R_{Xyl} 0.13, 0.08 and 0.015 (solvent A). This fraction was not further investigated.

3. DISCUSSION

3. 1 Anatheca dentata

Anatheca dentata (Suhr) Papenf., a red seaweed belonging to the Solieriaceae, was collected at Kowie Point (ca 120 miles east of Port Elizabeth) in March, 1967. It is fairly prominent along the coast of Southern Africa, occurring at the lowest level of the intertidal range.

Hot-water extraction of the fresh weed, followed by centrifugation, and precipitation of the mucilage into ethanol, afforded a highly sulphated polysaccharide mixture. This crude polysaccharide was purified by dissolution in water, centrifugation of the solution, and precipitation into ethanol. Complete acid hydrolysis of the polysaccharide mixture, followed by separation of the components on a cellulose column using half-saturated butanol, led to the isolation of galactose as a mixture of D- and L-isomers (1.57:1), and of D-xylose. These sugars were obtained in crystalline form and were characterised by their optical rotations, melting points, and mixed melting points with authentic samples. The galactose and D-xylose were further characterised by conversion to mucic acid and the O-dibenzylidene dimethyl acetal, respectively. Paper chromatographic evidence was obtained for the presence of traces of glucose, mannose, arabinose, and a sugar having chromatographic mobility of R_{Gal} 2.91 (solvent B). In addition, the hydrolysate contained a sugar with the mobility of R_{Gal} 2.1 (solvent B), which was subsequently isolated from a partial acid hydrolysate of the galactan sulphate and characterised as 3-O-methylgalactose.

Fractionation of the polysaccharide mixture with Cetavlon yielded two fractions. The minor one, which was not precipitated with Cetavlon, was composed of glucose and mannose residues, while the Cetavlon-precipitated major fraction contained chiefly galactose, xylose and ester sulphate. The sugar residues of the major fraction were quantitatively estimated as their glycitol acetates, using a gas chromatographic method devised by Bowker and Turvey[84] (Table I).

TABLE 1

COMPARISON BETWEEN CETAVLON-PRECIPITATED POLYSACCHARIDE AND FRACTION 2 (EX DEAE-SEPHADEX A-50 COLUMN)

	CETAVLON-PRECIPITATED POLY-SACCHARIDE	FRACTION 2 (EX DEAE-SEPHADEX A-50 COLUMN)
$[\alpha]_D^{21}$	-40.5° (c 0.99)	-33° (c 0.96)
SO_4^{2-} (%)	35.5	35.2
$NaSO_3^-$ [A] (%)	38.1	37.8
GALACTOSE [B] (%)	59.0	57.0
XYLOSE [C] (%)	8.2	7.7
MOLAR RATIO OF A:B:C	6.78:6.01:1.00	6.96:6.00:0.97
3-O-METHYLGALACTOSE	trace	trace

The quantitative analysis of mixtures of monosaccharides by g.l.c. requires that a volatile derivative be preparable in quantitative yield from each monosaccharide and that these derivatives be resolved completely. Though a number of possible derivatives fulfil these

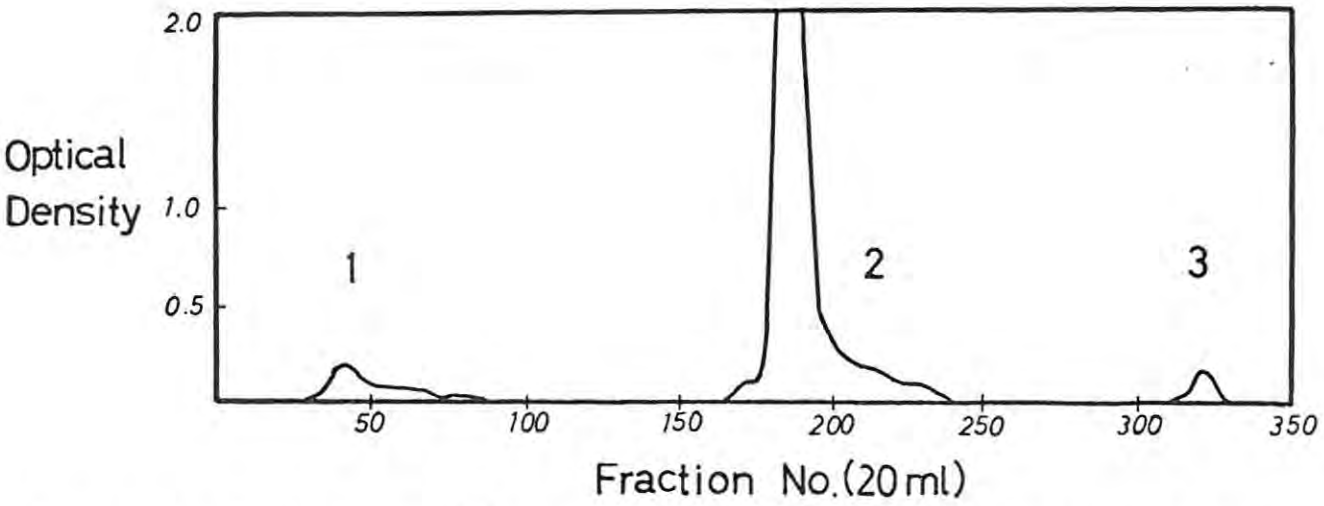


Fig. 1. Fractionation of Cetavlon-precipitated polysaccharide on DEAE-Sephadex A-50.

requirements, the main difficulty in working with monosaccharides is the formation of as many as four glycosides per monosaccharide as a result of anomeric and ring isomerization; each of these glycosides produces a peak in the chromatogram. Alditol acetates were chosen for these quantitative estimations as they cannot anomerize and, thus, the problems associated with multiple peaks are eliminated.

Chromatography of the Cetavlon-precipitated polymer on the anion exchange gel, DEAE- Sephadex A-50, gave three fractions (Fig. 1). All three fractions yielded galactose and xylose in approximately the same proportions on hydrolysis. The properties and molar ratio of the component sugars of the major fraction (fraction 2) were found to be very similar to those of the Cetavlon-precipitated polymer (Table I). Consequently all subsequent experiments were performed on the Cetavlon-precipitated polymer. It is most probable that the last fractionation effected a chiefly molecular weight separation, rather than a separation into structurally different polysaccharides. Although the Cetavlon-precipitated polysaccharide was not entirely homogeneous, there was no evidence from these experiments of a separate xylan. The infrared

INFRARED SPECTRA OF (a) SULPHATED POLYSACCHARIDE AND (b) DESULPHATED POLYSACCHARIDE

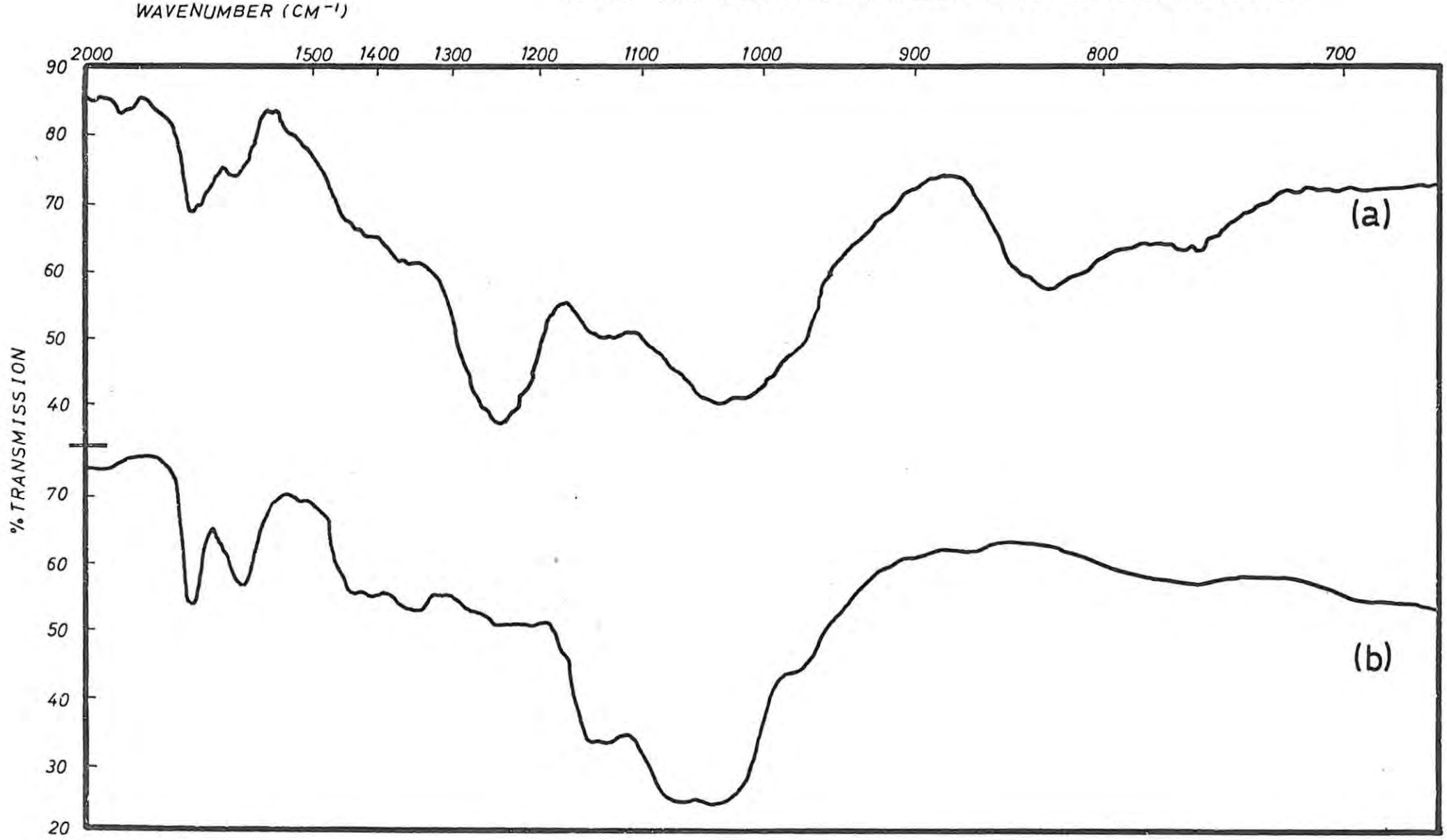


Fig. 2.

spectrum of the polymer (Fig. 2) exhibited the general absorption band for ester sulphate at 1240 cm^{-1} but did not show any well defined bands[5, 21] for axial, equatorial, or primary ester sulphate (800-860cm^{-1}).

Ether extraction of an acid hydrolysate of the polysaccharide yielded a residue which on treatment with 2, 4-dinitrophenylhydrazine, followed by separation by t. l. c., gave a derivative which was identical with the 2, 4-dinitrophenylhydrazone of pyruvic acid (mixed melting point and infrared spectrum). The presence of pyruvic acid in polysaccharides from red seaweeds was first demonstrated by Hirase[112] in 1957 when he isolated it as the 2, 4-dinitrophenylhydrazone from an acid hydrolysate of commercial agar. Subsequently, the isolation[113] and characterisation[114] of 4-O- β - [4, 6-O (1′-carboxyethylidene)-D-galactopyranosyl]-3, 6-anhydro-L-galactose dimethyl acetal from a methanolysate of commercial agar demonstrated its mode of attachment to the macromolecule. More recently Yaphe *et al*[115] have demonstrated that pyruvate is a common component of agarophytes. Pyruvic acid has also been isolated from the sulphated polysaccharide of *Phyllymenia cornea* (*Grateloupiaceae*).[116] In addition, there is evidence that it is present in the sulphated polysaccharides of other members of the *Grateloupiaceae*, namely *Aeodes ulvoidea* and *Aeodes orbitosa*.[116] The presence of pyruvate in these polymers suggests that pyruvate may well be of more widespread occurrence in red algal polymers than previously thought.

The pyruvic acid content of the polysaccharide has so far not been estimated. The usual method for determining the pyruvic acid content of polysaccharides is the procedure of Sloneker and Orentas[117] using 2, 4-dinitrophenylhydrazine after releasing the pyruvic acid with dilute hydrochloric acid. Values obtained by this method are at best only approximate since acid hydrolysis degrades polysaccharides to carbonyl

compounds (for example, 3, 6-anhydrogalactose residues yield keto acids, and pentoses yield furfural) which give a positive pyruvic acid reaction. Absolute values for pyruvic acid have been obtained[118] using the definitive lactate dehydrogenase method.[119] It is possible that methanolysis of the polysaccharide followed by g. l. c. examination of the products could be developed as a simple quantitative method for the estimation of pyruvic acid. Methanolysed pyruvic acid gives essentially one discrete peak on g. l. c. under the conditions described and, with a suitable internal standard, the pyruvic acid content could be determined from a standard calibration curve.

The elimination of sulphate ester groups from monosaccharide and polysaccharide sulphates in alkaline solution is well established.[28, 36] A sulphate ester group on position-6 of a galactose unit with a free hydroxyl group on position -3, or vice versa, is eliminated on treatment with alkali, with concomitant 3, 6-anhydride formation. In addition, a sulphate ester group situated on a secondary hydroxyl group is alkali-labile if there is an adjacent trans free hydroxyl group present.[38, 58] 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. Treatment of the sulphated polysaccharide from Anatheca dentata with alkali, in the presence of sodium borohydride[36] to prevent end-group degradation, led to the elimination of only 0. 85% of the sulphate, with negligible increase in the 3, 6-anhydro content of the polysaccharide. The polymer was retreated with alkali and the sulphate content was reduced by a further 1. 1% while the 3, 6-anhydro content increased from 0. 70% to 1. 31%. Thus, only a small amount of the sulphate in the polymer is suitably situated for 3, 6-anhydride formation. In addition, no

monomethylpentose or - hexose was formed when the polysaccharide was refluxed with sodium methoxide, which suggests the absence of additional alkali-labile sulphate groups.

Methylation of polysaccharides before and after desulphation has been used[120] to give information about the location of sulphate ester groups. An attempt was made to methylate the polysaccharide but due to its high sulphate content a maximum methoxyl content of only 10.15% was achieved presumably due to steric hindrance. The partially methylated polymer showed a large hydroxyl peak in its infrared spectrum, and was not further investigated.

In order to determine the most effective conditions for desulphation, samples of the polysaccharide were treated with 0.1M and 0.15M anhydrous methanolic hydrogen chloride[8] for various lengths of time. Treatment of the polysaccharide with 0.15M methanolic hydrogen chloride at room temperature for 48 hours, followed by isolation of the insoluble material and further treatment with 0.15M methanolic hydrogen chloride was found to be the most effective method. After five treatments, the polysaccharide (in 59% yield) had a sulphate content of 1.2%. A certain amount of glycosidic cleavage occurred during this process but it did not appear to be specific, since paper chromatography of an acid hydrolysate of the methanol-soluble material revealed galactose and xylose in approximately the same ratio as that found in the original polymer. The infrared spectrum of the desulphated polymer had no absorption bands in the 800-860 cm^{-1} region or at ca 1240 cm^{-1} (Fig. 2).

Oxidation of the polysaccharide with periodate was followed titrimetrically[87] and ceased after 96 hours at room temperature when 0.277 mole of periodate had been consumed per C_6- anhydro unit (Table II a). The resulting oxopolysaccharide was reduced with

borohydride. Complete acid hydrolysis of the polyalcohol, followed by paper chromatography, revealed the presence of galactose only. G.l.c. examination of the acetylated glycitols prepared from the hydrolysate confirmed the absence of xylose. The xylose residues in the polysaccharide must,therefore, be either 1,4-linked, 1,2-linked and/or present as a non-reducing end-group.

For comparative purposes, the polysaccharide and the desulphated polysaccharide were independently oxidised with periodate, the reaction being followed spectrophotometrically.[88] Oxidation of the sulphated polysaccharide ceased after 72 hours when 0.243 (0.377) mole of periodate had been consumed per C_6-anhydro unit (sulphate-free anhydrohexose unit). In the case of the desulphated polymer no definite end point was reached, even after 96 hours. At this stage, the rate of oxidation was extremely low and the desulphated polysaccharide had consumed 0.718 (0.726) mole per C_6-anhydro unit (sulphate-free anhydrohexose unit) (Tables II a and II b). The reduction of periodate in terms of a "C_6-anhydro unit" (calculated on the assumption that the polysaccharide is a homohexan) is usually chosen for simplicity but this concept is only of value for comparing the periodate uptake by polysaccharides of similar composition. To compare sulphated and desulphated polysaccharides, the periodate reduced per sulphate-free anhydrohexose unit[121] is much more meaningful, as allowance is made for the different sulphate content of these polysaccharides. The reduction of periodate (calculated on a sulphate-free basis; Table II b) by the desulphated polysaccharide is almost twice that of the sulphated polysaccharide. Desulphation of the polymer thus results in the production of new α-glycol groups.

TABLE II a

MOLE PERIODATE REDUCED PER C_6-ANHYDRO UNIT

Time(h)	4	12	24	48	72	96	120	144
Polysaccharide [a]	0.144	0.212	0.223	0.248	0.263	0.276	0.277	0.277
Polysaccharide [b]	0.047	0.084	0.168	0.189	0.243	0.243		
Desulphated [b] Polysaccharide (SO_4^{2-}, 1.2%)	0.382	0.549	0.597	0.690	0.711	0.718		

[a] Titrimetric determination [b] Spectrophotometric determination

TABLE II b

MOLE PERIODATE REDUCED PER SULPHATE-FREE ANHYDROHEXOSE UNIT

Time (h)	4	12	24	48	72	96
Polysaccharide [b]	0.073	0.130	0.260	0.293	0.377	0.377
Desulphated [b] polysaccharide (SO_4^{2-}, 1.2%)	0.387	0.556	0.604	0.698	0.720	0.726

[b] Spectrophotometric determination

Methylation of the desulphated polysaccharide was effected by repeated treatment of a solution of the polymer in dimethyl sulphoxide with solid sodium hydroxide and dimethyl sulphate. This yielded a chloroform-soluble gum having a methoxyl content of 36.0%. The chloroform-insoluble material gave on hydrolysis the same products as did the chloroform-soluble gum, but it could not be rendered chloroform-soluble on remethylation, and was not further investigated. The chloroform-soluble gum, after three treatments with Purdie's reagents,[89] had a methoxyl content of 40.3%. The infrared spectrum of this product showed a very small hydroxyl peak. Further treatment with Purdie's reagents[89] failed to increase the methoxyl content. The methylated desulphated polysaccharide was hydrolysed and the products separated by elution from a charcoal-Celite column with a linear gradient of 0-3% methyl ethyl ketone in water. The methylated galactoses obtained were 2, 3, 4, 6-tetra-O-methyl-DL-galactose, 2, 3, 6-tri-O-methylgalactose (predominantly as the L-isomer), 2, 4, 6-tri-O-methyl-D-galactose, a di-O-methyl fraction, a mono-O-methyl fraction and a small quantity of 2, 3, 4-tri-O-methyl-DL-galactose. The 2, 3, 6- and 2, 4, 6-tri-O-methylgalactoses were obtained in the approximate ratio of 2:1. The isolation of 2, 3, 6-tri-O-methylgalactose predominantly as the L-isomer indicates that most, if not all, of the L-galactose occurs as 1, 4-linked units in the polymer. However, some of the D-galactose is also 1, 4-linked. On the other hand, the isolation of 2, 4, 6-tri-O-methyl-D-galactose indicates that the unbranched 1, 3-linked galactose units have the D-configuration. The presence of 2, 3, 4, 6-tetra-O-methyl-DL-galactose in the hydrolysate implies that both D- and L-galactose occur as non-reducing end-groups in the desulphated polymer. The di-O-methyl

fraction contained 2, 6-di-O-methylgalactose (major sugar; predominantly the D-isomer), 2, 3-di-O-methylgalactose, 2, 4-di-O-methylgalactose and 4, 6-di-O-methylgalactose (trace). The mono-O-methyl fraction consisted of 2-O-methylgalactose (major sugar; predominantly the D-isomer), 4-O-methylgalactose and 6-O-methylgalactose. It is difficult to determine whether these products are the result of undermethylation or demethylation during hydrolysis or whether they have structural significance. However, their occurrence in fairly large quantities suggests that the polysaccharide is branched to some extent and that the branch points are mainly on D-galactose units. The 2, 3, 4-tri-O-methyl-DL-galactose is considered to have arisen from demethylation of some of the 2, 3, 4, 6-tetra-O-methyl-DL-galactose during hydrolysis.

As far as the xylose is concerned, the major methylated product was the 2, 3, 4-tri-O-methyl derivative; minor amounts of all three possible di-O-methylxyloses were also obtained. The latter are considered to be undermethylation products, a result supported by the observation that all the xylose units in the polymer were cleaved by periodate. These results, together with the fact that xylose was not encountered in any of the oligosaccharides obtained on partial acid hydrolysis (see later), indicate that the xylose occurs as a non-reducing end-group. A small amount of uronic acid was detected in acid hydrolysates of the polysaccharide and its presence was confirmed by the isolation of 2, 3, 4-tri-O-methyl-D-glucuronic acid from the hydrolysate of the methylated desulphated polymer. In addition, a minute amount of a second methylated uronic acid was detected in the hydrolysate. From the quantity of 2, 3, 4-tri-O-methyl-D-glucuronic acid obtained, it was estimated that the sulphated polysaccharide contains ca 3% by weight of D-glucuronic acid and, since it was isolated as the

2, 3, 4-tri-O-methyl derivative, it must occur (at least, in the desulphated polymer) as a non-reducing end-group.

Partial acid hydrolysis of the polysaccharide, followed by separation of the neutral products on a charcoal-Celite column yielded a mixture of D- and L- galactose, D-xylose, a trace of glucose, a small amount of 3-O-methylgalactose and several oligosaccharides. The 3-O-methylgalactose was identified by comparison of its chromatographic mobility on paper with that of an authentic sample, by demethylation[76] with hydrobromic acid to galactose, and by g. l. c. examination of the derived glycitol acetate when a peak having the same retention time as 1, 2, 4, 5, 6-penta-O-acetyl-3-O-methylgalactitol was observed. The low value obtained for the optical rotation suggests that the 3-O-methylgalactose is a mixture of the D- and L-isomers. The major oligosaccharide was 4-O- β -D-galactopyranosyl-L-galactose (1), followed in smaller amounts by 3-O- α -L-galactopyranosyl-D-galactose (2), 4-O-β -D-galactopyranosyl-D-galactose(3), O- β -D-galactopyranosyl (1→4)-O - α -L-galactopyranosyl (1→3)-D-galactose(4) and O- β -D-galactopyranosyl (1→4)-O- α -L-galactopyranosyl (1→3)-O-D-galactopyranosyl (1→4)-L-galactose (5). Oligosaccharides (1), (3), and (4) were obtained crystalline. Several other oligosaccharide fractions were obtained but in insufficient amount and purity for analysis. The structures of these compounds, except (3), which is known,[97] were obtained by the following series of experiments. The component sugars were determined by total and partial acid hydrolysis and the D- to L-galactose ratio was estimated from the optical rotation of each total hydrolysate. The reducing end-group was established by reduction of the oligosaccharide with sodium borohydride, followed by partial acid hydrolysis of the product and chromatographic examination

of the resulting mixture. The positions of the glycosidic linkages were established by methylation of the oligosaccharide followed by either methanolysis and examination of the resulting mixture by g. l. c., or hydrolysis followed by examination of the mixture by t. l. c. or paper chromatography. In those cases (1 and 2) where g. l. c. of the methyl-glycosides was used, quantitative estimation of the latter was effected. The results of these analyses, which are set out in Table III, are consistent with the structures of the oligosaccharides as formulated in the first column. The anomeric configurations of the glycosidic linkages assigned to the oligosaccharides were based on optical rotation. However, the author prefers not to speculate on the anomeric configuration of the remaining (1→4) glycosidic link in (5) because no trisaccharide with the composition O-L-galactopyranosyl (1→3)-O-D-galactopyranosyl (1→4)-L-galactose has been characterised from the partial hydrolysis products.

It is evident from the analysis of the oligosaccharides that a substantial part of the macromolecule must be composed of an alternating sequence of α-1, 3-D- and β-1, 4-L-galactose residues. Furthermore, because of the preponderance of D-galactose over the L-isomer in the polysaccharide, and because of the presence of 4-O- β -D-galacto-pyranosyl-D-galactose (3) amongst the partial hydrolysis products, D-galactose must replace some of the L-galactose units in the repeating sequence. The structure is complicated by the presence of xylose which has so far not appeared in any of the partial hydrolysis products. Periodate and methylation studies have, however, indicated that the xylose occurs as non-reducing end-groups, attached most probably to D-galactose residues in the macromolecule.

TABLE III

STRUCTURAL ANALYSIS OF NEUTRAL OLIGOSACCHARIDES

Oligosaccharide	Total Hydrolysis Products	Partial Hydrolysis Products	Partial Hydrolysis Products of Reduced Oligosaccharide	Hydrolysis or Methanolysis Products of Methylated Oligosaccharide
$G_D\,\beta\,(1\rightarrow4)G_L$ (1)	DL-galactose	-	D-galactose	A:B = 1.0:1.02
$G_L\,\alpha\,(1\rightarrow3)G_D$ (2)	DL-galactose	-	L-galactose	A:C = 1.0:1.05
$G_D\,\beta\,(1\rightarrow4)G_L\,\alpha\,(1\rightarrow3)G_D$ (4)	D:L-galactose ratio 2:1	(1), (2)	(1)	A, B, C
$G_D\,\beta\,(1\rightarrow4)G_L\,\alpha\,(1\rightarrow3)G_D(1\rightarrow4)G_L$ (5)	DL-galactose	(1), (2), (4)	(4)	-

A = 2, 3, 4, 6-Tetra-O-methylgalactose

B = 2, 3, 6-Tri-O-methylgalactose

C = 2, 4, 6-Tri-O-methylgalactose

The acidic components of the partial acid hydrolysis of the polysaccharide were fractionated on a charcoal-Celite column in order to remove inorganic and high molecular weight materials. Successive separations of the low molecular weight acidic fragments on paper yielded nine chromatographically homogeneous compounds, as their ammonium salts. The only sulphated sugars isolated were L-galactose 3-sulphate (I) and L-galactose 6-sulphate (II). These two components were characterised as follows: they both had a DP of 1; on hydrolysis they each gave galactose as the only reducing sugar; the molar proportion of ester sulphate to galactose was found to be approximately 1:1 in each case; and a portion of each was methylated separately, methanolysed and g. l. c. examination of the derived glycosides revealed peaks corresponding to those of methyl 2, 4, 6-tri-O-methyl- and 2, 3, 4-tri-O-methylgalactosides from the 3- and the 6-sulphate, respectively. The sulphated sugars both had negative optical rotations indicating the galactose was present as the L-isomer. From a study of the neutral fragments of the partial hydrolysate, it is apparent that the L-galactose units are linked through position-4. Sulphate on position -3 or -6 of such units would be alkali-labile. It follows that, since the sulphate groups in the polymer are all stable to alkali, those L-galactose residues carrying sulphate must either be the site of branch points, or be present as trisulphated residues. The latter units would not be expected to be alkali-labile since base hydrolysis (so far unrecorded in carbohydrate sulphates) of one of the sulphate groups would be necessary to produce the oxide ion required for the formation of 3, 6-anhydro, or 2, 3-epoxy derivatives. If, as it appears, all the xylose is present as non-reducing end-groups, each mole of xylose would consume two

moles of periodate. Thus, from periodate oxidation studies on the sulphated polysaccharide, ca 10% of the galactose units are cleaved by periodate. These galactose units must be 1, 4-linked and non-sulphated since 1, 3-linked galactose units are resistant to attack by periodate and a sulphate group on position-6 of a 1, 4-linked galactose residue would be alkali-labile. After desulphation, approximately half the galactose units were cleaved by periodate and therefore desulphation must have removed sulphate groups from position-2 and/or -3 of the remaining 1, 4-linked galactose units. It is of interest to note that the molar ratio of L-galactose to sulphate in the polymer is 1:3.

An aldobiuronic acid, identified as 4-O-α -D-glucuronosyl-L-galactose(III) was isolated from among the acidic fragments. On hydrolysis III gave galactose and glucuronic acid. Reduction of the carboxyl group of the uronic acid moeity and hydrolysis of a portion of the resulting neutral oligosaccharide, yielded L-galactose and D-glucose in the ratio 1.0:1.08. The remaining neutral oligosaccharide was methylated, methanolysed and the derived glycosides examined by g.l.c. Peaks corresponding to methyl 2, 3, 4, 6-tetra-O-methyl-glucosides and methyl 2, 3, 6-tri-O-methylgalactosides were obtained. A further portion of the aldobiuronic acid was methylated keeping the carboxyl group of the uronic acid moeity intact. After reduction of the carboxyl group, the product was methanolysed and gave peaks corresponding to methyl 2, 3, 4-tri-O-methylglucosides and methyl 2, 3, 6-tri-O-methylgalactosides on g.l.c. The α-configuration of the glycosidic linkage of III was assumed from its specific rotation. The β -linked aldobiuronic acid would be expected to have a negative specific rotation. A small amount of free glucuronic acid (VII) was also obtained from the partial hydrolysate.

Three pyruvate-containing saccharides were isolated, two of which were identified as 4, 6-O (1′-carboxyethylidene)-D-galactose (IV) and 4-O-β-[4, 6-O (1′-carboxyethylidene)-D-galactopyranosyl]-L-galactose (V). The structures were elucidated as follows: the component sugars were determined by total and partial acid hydrolysis; the presence of pyruvic acid confirmed by g. L. c. examination of a methanolysate of each saccharide; and the positions of the glycosidic linkage and of attachment of the pyruvic acid determined by methylation of each sugar followed by g. l. c. examination of the derived methyl glycosides and/or glycitol acetates. The third pyruvate-containing sugar (VI) is a O-[4, 6-O (1′-carboxyethylidene)-galactopyranosyl]-galactose but insufficient material was available for complete structural elucidation.

Two further acidic sugars (VIII and IX) were isolated in very small amounts from among the partial acid hydrolysis fragments. Both sugars were devoid of sulphate and were unchanged by acid hydrolysis.

It is evident from the studies carried out so far that the sulphated polysaccharide from Anatheca dentata is a highly complex polymer, and more information is required before a unique structure can be proposed. The results obtained indicate that the polymer is basically a galactan having an alternating sequence of α-1, 3-D- and β-1, 4-L-galactose residues as well as several unusual features, namely, xylose branches or end-groups or both, a distinct possibility of disulphated and/or trisulphated L-galactose units and some pyruvate residues. That the xylose is present as a non-reducing end-group proves that it is part of the heteropolysaccharide and not present as a separate xylan.

Thus, the sulphated polysaccharide from Anatheca dentata would appear to conform to the agarose-type structure[1, 122] (alternating α-1, 3-D- and β-1, 4-L-galactose units) as, for example, in agar and porphyran. In agar, the 1, 4-linked L-galactose units occur as the 3, 6-anhydride while in porphyran, only part of the 1, 4-linked L-galactose units occur as the 3, 6-anhydride, the remainder being present as the 6-sulphate. In comparison, the sulphated polysaccharide from Anatheca dentata contains a negligible amount of 3, 6-anhydrogalactose and although the L-galactose residues are sulphated, the sulphate is alkali-stable. However, the polysaccharide has not a homogeneous agarose-type structure as there are regions of adjacent D-galactose residues, i. e. in part it has a carrageenan-type structure [1, 122] (alternating α-1, 3-D- and β -1, 4-D-galactose units). It is interesting to note that polysaccharides from Eucheuma species,[123] which belong to the same family as Anatheca dentata (the Solieriaceae), structurally resemble the carrageenans. The extract of Eucheuma cottonii is similar to κ -carrageenan in composition and physical properties, while the extract of Eucheuma spinosum has a sulphate content approaching that of λ-carrageenan, but a 3, 6-anhydride content like that of κ -carrageenan.

3. 2 Chaetangium erinaceum

Chaetangium erinaceum (Turn.) Papenf., a red seaweed belonging to the Chaetangiaceae, was collected at Port Alfred (Indian Ocean) in October, 1966. It is a striking seaweed with thin flat fronds bearing many, branching outgrowths and is often found with Gelidium pristoides on exposed surfaces of rocks and wave-cut platforms in the mid-tidal range.

Hot-water extraction of the fresh weed, after careful separation from contaminating Gelidium pristoides, afforded a mucilage which was precipitated into ethanol. The crude polysaccharide (7.3% on a wet wt. basis) was shown by paper chromatography to contain xylose (major sugar), mannose and galactose. Fractionation of the polysaccharide mixture with Fehling's solution yielded two fractions, a xylan (ca 70%) and a sulphated polysaccharide fraction. The xylan was purified by further treatment with Fehling's solution and finally by dissolution in water, centrifugation of the solution, and precipitation into ethanol. All subsequent experiments were carried out on the xylan.

The xylan had a specific rotation of -112^{o}, indicating a predominance of β-linkages. Oxidation with periodate in unbuffered solution at room temperature ceased after 96 h (Table IV), when 0.812 mole of periodate had been consumed per C_5-anhydro unit.

TABLE IV

MOLE PERIODATE REDUCED PER C_5-ANHYDRO UNIT

Time (h)	5	12	24	48	72	96	120
xylan	0.594	0.664	0.712	0.760	0.800	0.812	0.813

Thus ca 80% of the xylose residues possess α-glycol groups and these are linked, therefore, through C1 and C2 or C4, if the possibility of the presence of furanose residues is discounted. The resistance to attack of the remaining 20% can be explained only by the presence of 1, 3-linkages.

Methylation of the polysaccharide by the method of Srivastava et al[103] afforded a partially methylated product which was fractionated on a silica gel column by elution with chloroform-methanol. Four fractions were obtained, all of which gave very similar patterns of methylated sugars on hydrolysis, the fractions with lower methoxyl values having larger quantities of mono-O-methylxyloses and unmethylated xylose (Table V).

TABLE V

METHYLATED POLYSACCHARIDE FRACTIONS EX SILICA GEL COLUMN

Fraction	A	B	C	D
Eluting solvent $CHCl_3$-Methanol	100:0, 610ml	80:20, 700ml	75:25, 300ml	60:40, 300ml
Weight (g)	0.772	3.62	0.463	0.222
OMe, %	35.7	34.9	23.4	20.2
Hydrolysis products: xylose	-	minute trace	trace	+
Mono-O-methyl-xyloses	trace	trace	trace	+
2, 4-di-O-methyl-xylose 2, 3-di-O-methyl-xylose 2. 3, 4-tri-O-methyl-xylose	Present in approximately equal proportions			

The largest fraction (71% of the total recovered polymer) had a methoxyl content of 34.9%, which on treatment with Purdie's reagents[89] was increased to 38.3%. Infrared examination of this material revealed no hydroxyl peak (Fig. 3). Further treatments with Purdie's reagents failed to increase the methoxyl content. The methylated polysaccharide was hydrolysed using the sulphuric acid method of Garegg and Lindberg.[104] Because of the volatility of tri-O-methylpentoses, the procedure was modified in that the acid hydrolysate was extracted with dichloromethane before neutralisation of the aqueous phase with barium carbonate.[105] Dichloromethane was chosen because of its volatility and the associated ease of recovery of the extracted sugars. The products of hydrolysis of the methylated polysaccharide were separated by paper chromatography to give 2,3,4-tri-O-methyl-D-xylose, 2,3-di-O-methyl-D-xylose, 2,4-di-O-methyl-D-xylose and a mono-O-methyl fraction. The 2,3-di-O-methyl- and 2,4-di-O-methyl-D-xyloses were identified as crystalline derivatives while the 2,3,4-tri-O-methyl-D-xylose was identified on the combined evidence of paper and gas-liquid chromatography. The low specific rotation found for the 2,3,4-tri-O-methyl-D-xylose was considered to be due to contamination with degradation products arising during hydrolysis of the methylated polymer. These are extracted with dichloromethane and have a high mobility on paper (as does 2,3,4-tri-O-methyl-D-xylose, R_{TMG} 1.11 in solvent B).

Both the acetylated nitriles and alditol acetates are suitable for g.l.c. analysis of the O-methylxyloses derived from the methylated xylan.[105] The nitriles were selected for this investigation, because of the greater simplicity of the experimental procedure and

INFRARED SPECTRA OF (a) XYLAN
AND (b) METHYLATED XYLAN (METHOXYL, 38.3%)

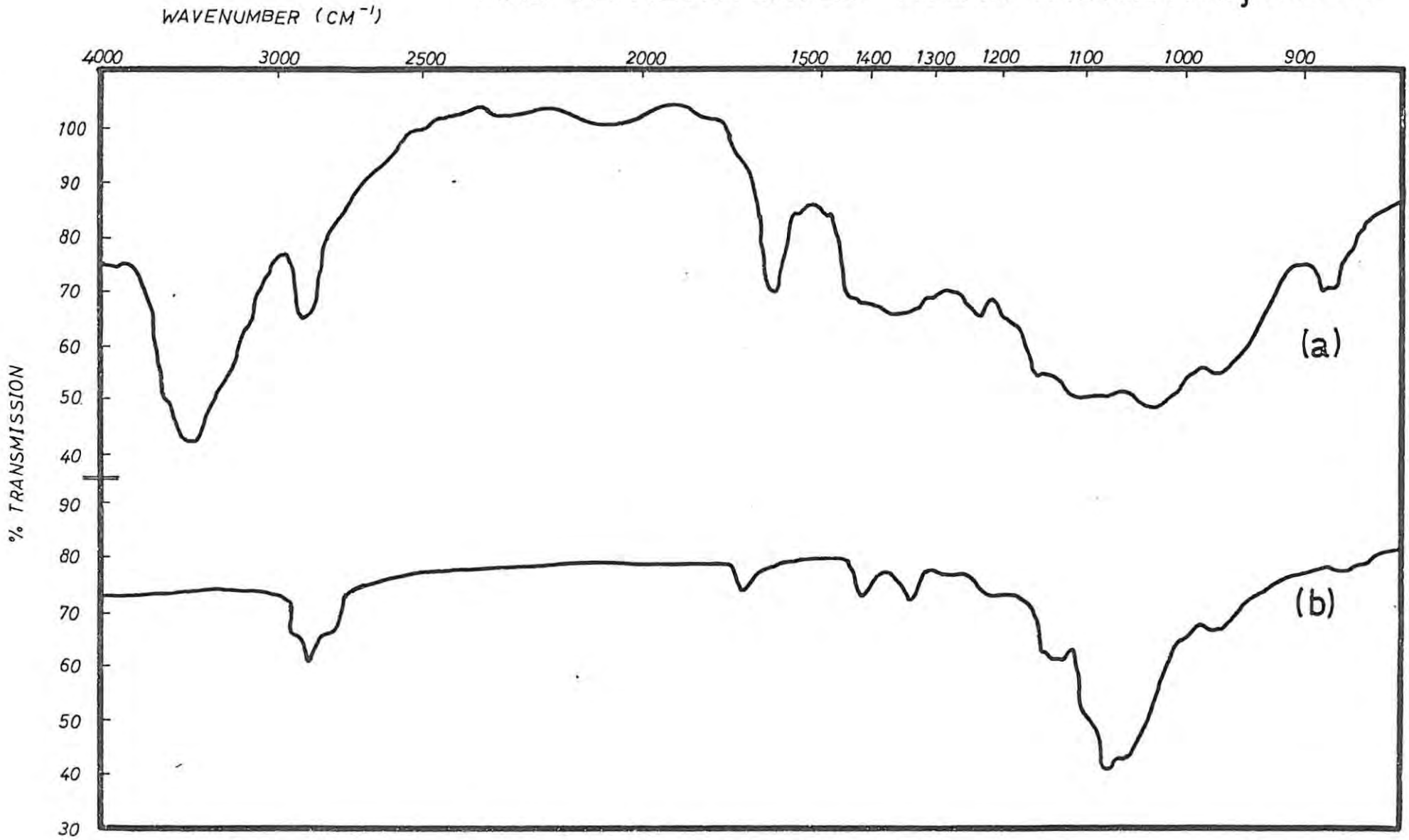


Fig. 3.

also because of the possibility of loss of 2, 3, 4-tri-O-methylxylitol during evaporation of the solution after neutralisation of the sodium borohydride with sulphuric acid. A sample of the methylated polysaccharide was hydrolysed and the derived monomers converted into their acetylated nitriles and examined by g. l. c. The relative molar ratio of 2, 3, 4-tri-: 2, 4-di-: 2, 3-di-O-methylxylose: mono-O-methylxyloses was shown to be 0. 08:1:4. 56: 0. 27. The amount of mono-O-methylxyloses was low; these ethers probably have no structural significance and are believed to be the result of undermethylation, and demethylation during hydrolysis. The methylation analysis, therefore, indicates that the xylan is essentially linear. The ratio between β-1, 4- and β-1, 3- linkages in the polysaccharide, as determined by methylation analysis, is in good agreement with the values estimated by periodate oxidation. The average chain-length of the xylan, estimated from the relative molar proportion of 2, 3, 4-tri-O-methyl-D-xylose, is about 74 xylose units. This estimate may, however, not be an accurate reflection of the length of the xylan chain due to the volatility of the 2,3, 4-tri-O-methyl-D-xylose, although all possible precautions to counteract this were taken during the analytical determination. The relative molar ratio of the O-methylxyloses isolated by paper chromatographic separation of the hydrolysed methylated polymer, agrees fairly well with the ratio obtained by g. l. c. analysis (see Table VI).

Partial, acid hydrolysis of the polysaccharide, followed by separation of the products on a charcoal-Celite column, yielded D-xylose and several oligosaccharides. The D-xylose was obtained in crystalline form and was characterised by its melting point, mixed

TABLE VI

MOLAR% O-METHYLXYLOSES FROM METHYLATED XYLAN

	2, 3, 4-Tri-O-methyl-xylose	2, 4-Di-O-methyl-xylose	2, 3-Di-O-methyl-xylose	Mono-O-methyl-xyloses
G. l. c. analysis	1. 35	16. 9	77. 9	4. 5
Ex paper separation	3. 1	16. 3	74. 6	6. 0

melting point with an authentic sample, and optical rotation. It was further characterised by conversion to the O-dibenzylidene dimethyl acetal derivative. The structures of seven of the oligosaccharides were elucidated, these being xylobiose (1), rhodymenabiose (2), xylotriose (3), O- β -D-xylopyranosyl (1→3)-O- β -D-xylopyranosyl (1→4)-D-xylose (4), O- β -D-xylopyranosyl (1→4)-O- β -D-xylopyranosyl (1→3)-D-xylose (5), xylotetraose (6), and xylopentaose (8). Oligosaccharides (1), (3), (6) and (8) were obtained crystalline. The structures of oligosaccharides (1), (2), (3), (6) and (8) were elaborated by partial, acid hydrolysis to the component sugars and by methylation studies. A portion of each methylated oligosaccharide was methanolysed and the resulting mixture of methylated glycosides examined by g. l. c. The remainder was hydrolysed and the derived methylated sugars were converted into their acetylated nitriles and analysed by g. l. c. Oligosaccharides (4) and (5) were elucidated as above, but, in addition, they were reduced with sodium borohydride, partially hydrolysed, and the products examined by paper chromatography. This procedure established the nature of the reducing end-group. The acetylated nitriles from each methylated oligosaccharide, with the exception of

TABLE VII

STRUCTURAL ANALYSIS OF OLIGOSACCHARIDES

Oligosaccharide	Partial Hydrolysis Products	Partial Hydrolysis Products of Reduced Oligosaccharide	Acetylated Nitriles of Hydrolysed Methylated Oligosaccaride
$X_D\ \beta\ (1\rightarrow 4)X_D$ (1)	-		A:B = 1: 0.9
$X_D\ \beta\ (1\rightarrow 4)X_D\ \beta\ (1\rightarrow 4)X_D$ (3)	(1)		A:B = 0.9:2.2
$X_D\ \beta\ (1\rightarrow 4)X_D\ \beta\ (1\rightarrow 4)X_D\ \beta\ (1\rightarrow 4)X_D$ (6)	(1), (3)		A:B = 0.9:3.1
$X_D\ \beta\ (1\rightarrow 4)X_D\ \beta\ (1\rightarrow 4)X_D\ \beta\ (1\rightarrow 4)X_D\ \beta\ (1\rightarrow 4)X_D$ (8)	(1), (3), (6)		A, B
$X_D\ \beta\ (1\rightarrow 3)X_D$ (2)	-		A:C = 1:1.1
$X_D\ \beta\ (1\rightarrow 4)X_D\ \beta\ (1\rightarrow 3)X_D$ (5)	(1), (2)	(1)	A: B: C = 0.9 : 1.0 :1.1
$X_D\ \beta\ (1\rightarrow 3)X_D\ \beta\ (1\rightarrow 4)X_D$ (4)	(1), (2)	(2)	A:B:C = 1.1: 0.9: 1.0

A = 2, 3, 4-Tri-O-methylxylose
B = 2, 3-Di-O-methylxylose
C = 2, 4-Di-O-methylxylose

oligosaccharide (8), were quantitatively estimated. The results of these analyses, which are set out in Table VII, are consistent with the structures of the oligosaccharides as formulated in the first column. The type of glycosidic linkage assigned to the oligosaccharides was based on optical rotation or comparison with the optical rotation of authentic compounds.

"Oligosaccharide" (7), although chromatographically homogeneous in all solvents, was shown by electrophoresis to be a mixture of at least two components. Partial, acid hydrolysis studies, before and after treatment with sodium borohydride, indicated that it was probably a mixture of O-β-D-xylopyranosyl (1→4)-O-β-D-xylopyranosyl (1→4)-O-β-D-xylopyranosyl (1→3)-D-xylose, O-β-D-xylopyranosyl (1→4)-O-β-D-xylopyranosyl (1→3)-O-β-D-xylopyranosyl (1→4)-D-xylose, O-β-D-xylopyranosyl (1→3)-O-β-D-xylopyranosyl (1→4)-O-β-D-xylopyranosyl (1→4)-D-xylose, and/or O-β-D-xylopyranosyl (1→3)-O-β-D-xylopyranosyl (1→4)-O-β-D-xylopyranosyl (1→3)-D-xylose.

When log $(1/R_F-1)$ of the oligosaccharides present in the partial hydrolysis, calculated from a chromatogram in solvent A, is plotted against DP, the points fall on two nearly parallel straight lines (Fig. 4). The R_F values were calculated from the R_{Xyl} values of the oligosaccharides multiplied by the R_F of xylose (0.21). The upper line represents the oligosaccharides containing only 1,4-linkages and the lower line those containing both 1,4- and 1,3-linkages. It is of interest to note that the major component of Fraction IX (which was not structurally elucidated), R_{Xyl} 0.03 (solvent A), falls onto the lower line if it is assumed

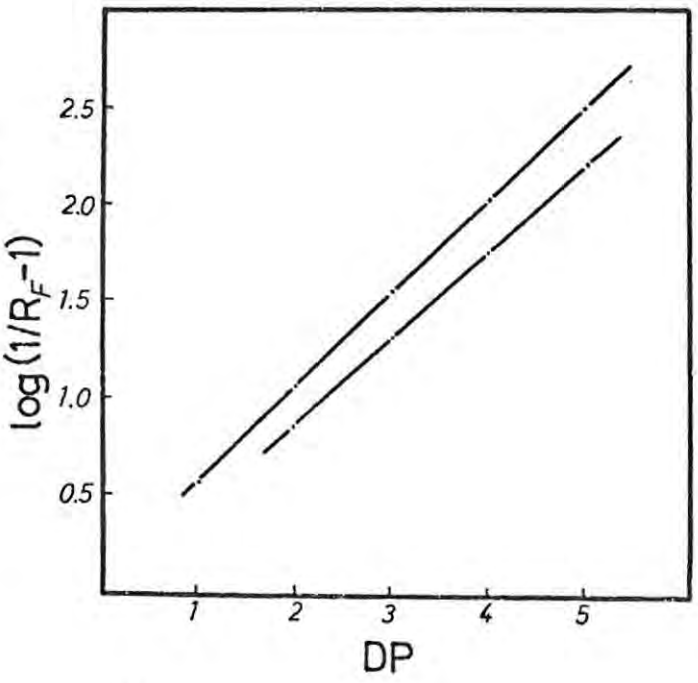


Fig. 4. Relationship between chromatographic mobility and DP of xylose oligosaccharides.

to be a mixture of pentasaccharides containing both 1, 4- and 1, 3-linkages.

The isolation in substantial yield of two pure trisaccharides containing both a 1, 4- and a 1, 3-linkage affords conclusive proof that the polysaccharide molecule does indeed contain both types of linkage. No substances were detected in the hydrolysate which could be the trisaccharide O-β-D-xylopyranosyl (1→3)-O-β-D-xylopyranosyl (1→3)-D-xylose or the tetrasaccharides O-β-D-xylopyranosyl (1→3)-O-β-D-xylopyranosyl (1→3)-O-β-D-xylopyranosyl (1→4)-D-xylose or O-β-D-xylopyranosyl (1→4)-O-β-D-xylopyranosyl (1→3)-O-β-D-xylopyranosyl (1→3)-D-xylose. The conclusion may tentatively be drawn that the xylan chain does not contain two adjacent 1, 3-linkages. However, as acid hydrolysis is known to favour the cleavage of 1, 3-links, degradation of the xylan with a process which selectively cleaves 1, 4-links (for example, an enzyme preparation) is necessary before any definite conclusion is drawn.

The oligosaccharides containing a 1, 3-linkage, although paper chromatographically more mobile than the corresponding oligosaccharides containing only 1, 4-linkages, were more firmly held on a charcoal-Celite column. This observation supports that made by Howard[67] in his investigation of the partial hydrolysis products of the xylan from Rhodymenia palmata.

Thus the xylan molecule is essentially linear and is composed of β 1, 3- and β1, 4- linked units in the ratio 2:9. There are regions of adjacent 1, 4-linkages interspersed with 1, 3-linkages but, as yet, no evidence has been obtained for contiguous 1, 3-linkages. It is, therefore, similar to the water soluble xylan from Rhodymenia palmata[59] which contains ca 80% β1, 4- and 20% β1, 3-linkages. It is interesting to note, however, that whereas the water-soluble fraction of Rhodymenia palmata consists entirely of xylan, the xylan from Chaetangium erinaceum makes up only part of the water-soluble extract, the rest being a sulphated polysaccharide mixture. In this respect, the resemblance to the Chaetangium fastigiatum xylan[75] is quite marked. Both Chaetangium erinaceum and Chaetangium fastigiatum belong to the order Nemalionales. The hot-water extract from Chaetangium fastigiatum yielded a polysaccharide mixture which on hydrolysis gave xylose (major), galactose, mannose and ester sulphate. The acidic polysaccharide was removed by precipitation with cetylpyridinium chloride, leaving a neutral xylan. Methylation analysis indicated that the xylan contains 1, 3- and 1, 4- linked β -D-xylopyranose residues in the approximate ratio 1:3. Rhodochorton floridulum,[59] another member of the Nemalionales, contains a polysaccharide which has 1, 3- and 1, 4- linked xylose residues in the approximate ratio 1:4, and a considerable degree of branching. It has been suggested that the xylose chains are short side branches on another polysaccharide backbone, probably a glucan.

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