"A STRUCTURAL INVESTIGATION OF THE SULPHATED POLYSACCHARIDE OF PACHYMENIA CARNOSA (J. AG.) J. AG."

by

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CONTENTS

SUMMARY

The highly sulphated, methylated polysaccharide isolated from Pachymenia carnosa, a red seaweed, was shown to contain D-galactose, 2-o-methyl-D-galactose, 6-o-methyl-D-galactose and 4-o-methylgalactose. The polysaccharide was desulphated with methanolic hydrogen chloride. Methylation of the desulphated polysaccharide revealed that it was composed entirely of (1→3) and (1→4) links in approximately equal amounts. Treatment of the polysaccharide with alkali showed that the majority of the ester sulphate groups were alkali-stable. Partial hydrolysis and acetolysis studies indicated that the polysaccharide was extremely complex, and contained alternate α(1→3) and β(1→4) glycosidic linkages. There is evidence for the presence of D-galactose-6-sulphate.

1. INTRODUCTION

SELECTED METHODS AND TECHNIQUES APPLIED TO POLYSACCHARIDE STRUCTURE ELUCIDATION

At present there is no single technique which will give all the information required for the complete structural elucidation of a polysaccharide. It is necessary to carry out various operations on the polysaccharide the results of which, when viewed as a whole, enable the structure to be determined.

The most important advance that could be made in structural polysaccharide chemistry would be the development of a method analogous to that in existence for proteins, whereby individual monosaccharides could be sequentially removed from the chain in a controlled manner. To a limited extent specific exo-enzymes have been used to remove the non-reducing terminal units of polysaccharides.[1] Application of exo-glycosidases of defined specificity can give considerable information on the structural arrangement of non-reducing terminal residues, and in particular, on the anomeric configuration of the interunit linkage. This method is, however, applicable only to specific types of structures being primarily effective with a highly branched molecule, and only of limited use with a linear polymer.

The following is a critical review of some of the general methods and techniques which are frequently used for polysaccharide structural elucidation. Several of the techniques described have been used in the structural investigation of Pachymenia carnosa, which is the subject of this thesis.

1. 1 Methylation

Methylation is one of the most important techniques in structural polysaccharide chemistry. The procedure involves the preparation of an exhaustively methylated polysaccharide, hydrolysis of this to a mixture of monomers, and the separation, identification and quantitative estimation of the components of this mixture. The original points of substitution will correspond to the unsubstituted hydroxyl groups in the monomeric methyl ethers.

Polysaccharide methylation began with the methylation, using methyl sulphate, of alkali-cellulose impregnated with a 15% solution of sodium hydroxide.[2] This procedure of Denham and Woodhouse, modified by Haworth,[3] who added the aqueous sodium hydroxide (ca 30%) and the methyl sulphate to an aqueous solution of the polysaccharide, remains a standard method. Fresh reagents are usually added to the reaction mixture after the first methylation period with, or without, removal of the sodium sulphate formed, and the process repeated until there is no further increase in methoxyl content. The operation may be carried out in an atmosphere of nitrogen so as to keep alkaline degradation at a minimum.

The above method does not, in general, give a completely methylated polymer. Methylation is usually completed by treatment with silver oxide in gently boiling methyl iodide.[4] The reaction is performed under anhydrous conditions and the silver oxide added in portions with continual vigorous stirring. This method is not always very effective and has, generally, to be repeated for exhaustive methylation. It is particularly useful due to the ease of recovery of the methylated product. If an unmethylated polysaccharide is treated with Purdie's reagents extensive oxidative degradation occurs due to the strongly basic silver oxide. However, when most of the original hydroxyl groups are methylated,

or if the polysaccharide is reduced prior to methylation, the degradation is greatly reduced.

Oxidative degradation can be decreased by carrying out the reaction at reduced temperature, a method employed for the methylation of oligosaccharides.[5] The oligosaccharide, dissolved in N,N-dimethylformamide, is treated with methyl iodide and silver oxide at 0^{o}. The methylation can then be carried out at elevated temperature since the resultant methyl glycosides are resistant to the reagents. N,N-Dimethylformamide is utilized in the reaction since it aids dissolution of the oligosaccharide and also increases the rate of reaction.

The procedure of Freudenberg and co-workers[6] for methylating starch in liquid ammonia is very efficient. However, this method has been avoided in favour of the laborious methyl sulphate-alkali procedures because solutions of the ethers produced showed very low viscosities; indicating possible degradation of the polyglucose chains. Hodge et al[7] found it possible to limit the degradation of the polysaccharide chain by ensuring no excess sodium was present at any time. They found that sodium-ammonia caused no degradation, in fact degradation arose from the action of sodium amide which was formed in sodium-ammonia solutions on standing. Practically, this method tends to be a bit clumsy since at least part of the procedure is carried out at low temperature, and atmospheric moisture is absorbed if the reaction is not conducted in a closed vessel.

In addition to the classical techniques which often require many additions of reagent more recent methods have been reported to give improved yields with fewer treatments. One of these developed by Hakomori[8] involves treatment of the carbohydrate with the base methyl

sulphinyl methyl sodium in dimethyl sulphoxide, followed by reaction with methyl iodide.

The base may be prepared by adding sodium hydride to a solution of the carbohydrate in dimethyl sulphoxide.[9] The ionization of the hydroxyl groups in the polysaccharide by the base is a comparatively slow reaction[8] and the polysaccharide is, thus, left in contact with the base for several hours before addition of the methyl iodide. Ultrasonic treatment facilitates reaction between sodium hydride and dimethyl sulphoxide.[10] It is essential that the solution of methyl sulphinyl methyl sodium is protected from contact with air. The polyalkoxide ions react readily with methyl iodide, yielding a completely methylated product after only one treatment. When methylation is complete the reaction mixture is poured into water and the fully methylated polysaccharide isolated by dialysis or extraction.

Polysaccharides that do not dissolve or swell in dimethyl sulphoxide cannot be methylated by this technique; in which case the best procedure is initial partial methylation of the polysaccharide using a classical technique which can then be followed by a Hakomori procedure. This method of methylation is ideal for work on a micro scale.

With the intention of comparing methods of methylation[11] three different procedures were used to methylate the hemicelluloses of the tropical pasture species Cynodon plectostachyus and Setaria sphacelata; viz. (i) Haworth followed by the Purdie procedure, (ii) sodium hydride-dimethyl sulphoxide - methyl iodide[8] (completed by the Purdie method) and (iii) sodium hydroxide-dimethyl sulphoxide -methyl sulphate (the method of Srivastava and Singh[12]) completed by the Purdie method. The methanolysis products of the methylated polysaccharides were

examined by gas-liquid chromatography (g. l. c.) and the molecular weights by vapour phase osmometry. It was found that method (ii) is superior to (i) for the polysaccharides studied, in that yields for the former were much higher, and, in the case of Cynodon the molecular weight was markedly higher. With (iii) lower molecular weights and extra g. l. c. peaks indicated that some degree of degradation had occurred, hence this method is unsuitable for such polysaccharides, at least under the conditions described.

When the native polysaccharide contains methylated monosaccharides these lose their identity on methylation and their structural features have to be found by other methods; for example, investigation of oligosaccharides, containing these sugars, obtained by partial hydrolysis of the polysaccharide. Another approach is to methylate the native polymer with trideuteriomethyl iodide.[13] Examination of the methylated sugars obtained on hydrolysis of the methylated polymer by g. l. c. -mass spectrometry permits the original and the introduced methoxyl groups to be distinguished.

Estimation of the completeness of methylation is usually done by a determination of the methoxyl content. However, methylated polysaccharides are hygroscopic and the last traces of dimethyl sulphoxide or other solvents are difficult to remove. Methoxyl analyses, thus, often give erroneously low values, and are impractical when only a few milligrams of polysaccharide are available. Another criterion of degree of methylation is the absence of absorbance in the 3400-3600 cm^{-1} region of the infra-red spectrum, the frequency region characteristic of hydroxyl groups; however, water also gives an absorbance peak in this region. If it has been established that the polymer is unbranched, the virtual absence of unmethylated and mono-methylated monomers from a hydrolysate of a methylated sample is a better criterion of exhaustive methylation.

In general, no further increase in degree of methylation with repeated treatments with a variety of methylating agents, can be taken to indicate

complete methylation. It is vital that the polysaccharide be as highly methylated as possible, since undermethylation could give rise to methyl ethers which might incorrectly be assumed to have structural significance.

In general, it is necessary to use a non-aqueous or only partially aqueous medium for the initial hydrolysis of methylated polymers, due to their insolubility in hot aqueous solutions. Several methods have been used, for example treatment with methanolic hydrogen chloride and subsequent hydrolysis of the methyl glycosides formed; heating with concentrated formic acid followed by hydrolysis of the formyl esters with aqueous mineral acid;[14] or prehydrolysis in concentrated (ca 70%) sulphuric acid at low temperature, with completion of hydrolysis by dilution and warming.[15] Some degradation and demethylation is unavoidable during the hydrolysis step,[16] the amount varying with the method used, for example hydrochloric acid usually causes more degradation than sulphuric acid. The best method appears to be an initial partial formylysis (about one hour reaction time) followed by complete hydrolysis with dilute sulphuric acid.

Mixtures of methylated sugars may be fractionated by partition chromatography on cellulose columns or by adsorption chromatography on charcoal-Celite columns using gradient elution.[17] The systems commonly used for elution are mixtures of water and ethanol or a low molecular weight ketone. It is often difficult to separate mixtures of highly methylated ethers by ordinary methods. Their R_F values are generally too high for good separation by partition chromatography, and on charcoal columns they are strongly adsorbed and are eluted only at high concentration of ethanol or ketone (and then with considerable trailing and incomplete separation). It is possible to separate highly

methylated monomers by column chromatography if carefully controlled conditions are used. Thus Nunn and Parolis[18] managed to obtain perfect separation of 2, 3, 6 - tri -o - methylgalactose from 2, 4, 6 - tri - o - methylgalactose off a charcoal-Celite column with gradient elution using aqueous methyl ethyl ketone by utilizing an abnormally large column and a very slow gradient. G.l.c. has been used for the separation of methylated glycosides and is especially useful for the characterisation of the monomers obtained.

Care must be taken in interpreting the products of a methylation. In general, a free hydroxyl group after exhaustive methylation and hydrolysis indicates a glycosidic linkage and more than one free hydroxyl group, a branch point. In the methylation of a sulphated polysaccharide free hydroxyl groups indicate either a glycosidic linkage or the position of a sulphate ester. The position of the latter can be determined by difference from methylation of a sulphate-free sample in addition to the native polymer.

This technique was used by Dolan and Rees[19] in investigating the structure of λ -carrageenan. The position of the glycosidic linkages was determined by methylation of a desulphated sample. The sole methylation products were 2, 3, 4, 6 - tetra - o - methyl - D - galactose and, in similar yields, 2, 4, 6 - tri - o - methyl - D - galactose, indicating the presence of 1,3 - linkages and 2, 3, 6 - tri - o - methyl - D - galactose which could have arisen from 1, 4 - or 1, 5 - linked galactose units. The 1, 5 - linkage was considered unlikely because 3, 6 - anhydro - D - galactose was formed from these units by alkaline elimination of 6 - sulphate. Since in a 1, 5 - linked galactofuranose this is sterically impossible the 1, 4 - linkage was favoured. In order to obtain evidence for the location of the sulphate esters, λ-carrageenan was methylated

with the sulphate groups intact. Some desulphation at position-6 occurred as a result of the alkaline methylation conditions. The major component of the methylated hydrolysate was 3 - o - methyl - D - galactose (45-58%) indicating 1, 4 - linked -2, 6 - disulphate units. The remaining methyl ethers were characterised as 4, 6 - di - o - methyl - D - galactose (31%) indicating 1, 3 - linked galactose - 2 - sulphate units, 2, 4, 6 - tri - o - methyl - D - galactose (13%) and 2, 6 - di - o - methyl - D - galactose (8%) presumably arising from 1, 3 - linked galactose - 4 - sulphate.

This technique has its limitations since complete methylation of a highly sulphated polysaccharide is extremely difficult to achieve even under forcing conditions, presumably due to steric hindrance.

Highly methylated sugars are often volatile, especially tri - o - methyl pentoses, and care has to be taken that these are not lost during the hydrolysis and subsequent treatment of the methylated polysaccharide. Losses can be eliminated either by extraction of the highly methylated products with dichloromethane or a similar low boiling organic solvent before neutralization and concentration of the hydrolyzate,[20] or by methanolysation of the methylated polysaccharide and analysing the methyl glycosides obtained by g. l. c. without preceeding neutralization or concentration of the methanolysate.[21]

1. 2- Gas-liquid Chromatography

Gas-liquid chromatography is under the appropriate conditions a powerful analytical tool because of its high efficiency, rapid operation and the reliability of both the qualitative and quantitative results obtained. Unfortunately not all compounds are ameanable to treatment by g. l. c. as its overriding limitation is the requirement of volatility and thermal

stability of the compounds to be analysed. Compounds that can be distilled or sublimed, even under diminished pressure, are generally sufficiently volatile. However, any monosaccharide derivative containing more than two free hydroxyl groups may not be sufficiently volatile for application of g. l. c. The volatility of such compounds can be increased by substitution of the hydroxyl function with methyl, acetyl or trimethylsilyl groups. The necessity for thermal stability is more difficult to provide and yet is of extreme importance. Column temperatures range from 150 - 250° during analysis and the injection site is usually maintained at a temperature some 60° higher than that of the column. Oxidative and hydrolytic processes are not very important in the atmosphere of inert gas used as the mobile phase, but many carbohydrate derivatives are noted for their lability at high temperatures and for undergoing complex rearrangements under a variety of conditions. The possibility of modification during g. l. c. should, therefore, not be overlooked.

Before the advent of paper chromatography fully methylated monosaccharides were separated from each other by fractional distillation, and it is not surprising that these were the first carbohydrate derivatives on which g. l. c. was tested. This procedure was directly extended for the investigation of the fully methylated derivatives of di- and tri-saccharides. In general the methylated oligosaccharide is methanolysed for 6 h with methanolic hydrogen chloride. The glycosidic bonds are broken and a series of methylglycosides (usually 2 or 4) formed from each constituent monosaccharide - the number depending on the individual components, since it is possible to form α- and β-glycoside pyranoses and furanoses with certain sugars. These glycosides are readily separable and identifiable by g. l. c., enabling the position of the glycosidic link to be determined. G. l. c. of the

methyl glycosides from some sugars show only one peak, due either to lack of resolution under the conditions used, or to the formation of a single, pure anomer.

In order to investigate mixtures of monosaccharides a volatile derivative (which can be resolved by g. l. c.) has to be prepared in quantitative yield from each monosaccharide. Derivatives which fulfil these requirements are the fully methylated or acetylated methyl glycosides, acetylated monosaccharides or alditols and trimethylsilyl ethers of methyl glycosides, glycoses or alditols. The main difficulty in working with products of methanolysis is the formation of as many as 4 glycosides from each monosaccharide; which increases the complexity of the chromatogram. This problem may be circumvented by finding conditions under which the derivatives from different monosaccharides are separated, but anomers and the 2 ring forms from a single sugar are not resolved. This is very specific and tedious as conditions which apply in one case can not necessarily be utilized in another situation. A far superior method is the use of the corresponding alditols, because there are no ring forms and hence no anomers, each monosaccharide yielding a single peak. Gunner, Jones and Perry[22] were the first to report the successful separation of a mixture of fully acetylated alditols. By this development they extended the use of g. l. c. to the general analysis of monosaccharide mixtures. The glycose mixtures were quantitatively analysed by reduction with sodium borohydride to the corresponding glycitols, followed by conversion to their fully acetylated derivatives by treatment with acetic anhydride. Separations were carried out using a mixture of liquid phases. Since these preliminary investigations simple liquid phases which are

suitable for the separation of acetylated alditol mixtures have been developed.

In certain cases it is not possible to use only alditol acetate derivatives. For example, in the partially methylated xylose series the acetylated alditols have a limited use because of the symmetry of xylitol - thus 2 - o - methyl - D - xylose gives the same derivative as the 4 - o - methyl sugar. Under these conditions it is necessary to use a derivative which retains the individuality of the parent sugars and gives single, well separated peaks on g. l. c. A suitable derivative[23] for analysis was found to be the acetylated nitrile - prepared by making the sugar oxime, and without isolation, heating it in pyridine - acetic anhydride to effect simultaneous acetylation and dehydration.

The isolation of pure components from complex mixtures of methylated sugars can be a long and difficult procedure requiring repeated chromatographic separations with the result that the quantitative data necessary for precise interpretation of the results may not be meaningful. With the greater resolving power, faster operation and reliable quantitative data provided by g. l. c. it was anticipated[24] that the method would be useful in this area of carbohydrate chemistry and, indeed, that is where it has found its widest application. An additional advantage is the determination of components by measurement of a physical property instead of by reaction with a functional group. It may be expected, therefore, that g. l. c. will reveal cleavage products whose presence had not been indicated previously.

In the quantitative analysis of a mixture of methyl o - methyl glycosides, two precautions must be observed: (a) that no component is lost

preferentially before analysis, and (b) that all components in the mixture are sufficiently volatile to pass through the columns and be detected. The first precaution requires that extreme care be taken in preparing methyl glycosides of the methylated sugars. Evaporation of any solution containing fully methylated methyl glycosides must be done at atmospheric pressure to prevent loss of these very volatile products. The second point can easily be checked by paper chromatography of a separate sample of the methylated sugars to see if any mono-*O*-methyl or unsubstituted sugars are present.

One of the disadvantages of investigating methyl glycoside products of a methylation is that each sugar gives a mixture of two to four glycosides in fairly constant proportions. This sometimes aids identification but more often overlapping of peaks complicates the results. Transforming the methylated sugars obtained on hydrolysis of a fully methylated polysaccharide into the acetylated alditol derivatives and analysing this mixture by g. l. c. on ECNSS-M columns[25] complements the analysis of methyl glycosides. In certain cases separation is better than that obtained for methyl glycosides. Also, as each sugar gives a single peak, quantitative evaluation of the products is considerably facilitated.

Another useful application of g. l. c. in structural investigations of polysaccharides is in the analysis of products obtained after periodate oxidation. Periodate oxidation of a polysaccharide yields "polyaldehydes" which usually undergo profound decomposition when hydrolysed even in the cold. The corresponding "polyalcohol" which is readily obtained from the "polyaldehyde" by reduction with aqueous sodium borohydride in the cold, can be subjected to hydrolysis with boiling dilute mineral acid, with little or no decomposition, to give

cleavage products which can be separated by partition chromatography and determined quantitatively. Results obtained in this manner provide information concerning the nature and amount of glycosidic linkages in a polysaccharide, since if free hydroxyl groups are present at C-2 and C-3 of a hexopyranose residue erythritol or threitol is obtained (e. g. with a 1, 4 - linkage), and residues with C-3 and C-4 free yield glycerol (e. g. terminal residue or 1, 2 - or 1, 6 - linkage) and if no two adjacent hydroxyl groups are free (e. g. 1, 3 - linkage) the sugar will not be attacked by periodate. These products can all be analysed by g. l. c. as their acetate derivatives. The deductions that can be made from the results of an examination of the polyalcohols produced from a given polysaccharide do not necessarily permit a clear cut solution to a structural problem, but taken in conjunction with other experimental results, for example those from methylation, it is feasible to restrict greatly the number of structural possibilities e. g. In a polysaccharide composed of hexopyranose residues joined by 1, 6 - and 1, 4 - linkages the ratio of these two types of linkage would correspond to the mole ratio of the glycerol to erythritol (or threitol) obtained from the corresponding polyalcohol by hydrolysis.

Since many isomeric carbohydrate derivatives can be resolved by g. l. c. it is apparent that separations depend on steric factors as well as on differences of molecular weight or degree of substitution. Generalizations of behaviour have been attempted[26] but are of limited applicability since the order in which isomers are eluted depends on both the liquid phase and derivative being used. The soundest generalization exists between the structure and g. l. c. mobility of alditol acetates. It has been conclusively shown[27] that isomers having the largest number of ester groups on the same side of the planar zig-zag

carbon chain have the largest retention times; if two isomers have the same number of ester groups on the same side of the molecule then the one with those groupings closest together will have the larger retention time.

The number and variety of applications mentioned indicate the general utility of g. l. c. in carbohydrate chemistry. The greater resolving power and more rapid separations are distinct advantages of g. l. c. over other chromatographic techniques. Since separations are usually completed within 45 min. and samples required are small, the method is ideally suited to exploratory work, checking the purity of reaction products and rate studies. Probably the most important advantage of g. l. c. over other analytical methods is that precise quantitative data can be obtained. Reaction mixtures can be injected directly into the apparatus so no errors are incurred through loss of products by evaporation, filtration or adsorption onto a precipitate. Analysis by g. l. c. also avoids the errors inherent in paper chromatographic methods involving elution of components from the paper and their estimation by chemical or colorimetric methods. An added attraction of analysis by g. l. c. is the suitability of derivatives e. g. the trimethylsilyl ethers, for mass-spectral analysis which provides an excellent method for confirmation of skeletal arrangement.

1. 3 Mass Spectrometry

Mass spectrometry was first applied to carbohydrate derivatives in 1958 when Reed et al[28] reported the mass spectra of D - glucose, D - galactose, methyl α- and β-D - glucopyranosides and a number of disaccharides, thus demonstrating that mass spectrometry is useful

in the field of carbohydrate chemistry.

Mass spectral studies[29] are generally carried out on volatile derivatives of mono- and oligo-saccharides, such as methyl ethers, acetates and alkylidene derivatives. Consequently experimental conditions are ideal for the simultaneous use of g. l. c. and mass spectrometry. One characteristic feature of mass spectra of carbohydrate derivatives is the frequent absence of the molecular ion. The steric differences are occasionally reflected only by changing relative intensities of some of the peaks, and hence, the applicability of this method to stereochemical problems is fairly limited. On the other hand, the mass spectrum can give a ready solution to a wide variety of structural problems. For example : to determine whether the carbohydrate moeity is in the cyclic or acyclic form; pyranoid or furanoid form; an aldose or ketose; determination of the position of substituents e. g. hydroxyl groups in partly methylated monosaccharides, linkage of the monosaccharide residues in disaccharides, methylene group in deoxy sugars; and determination of the number of carbon atoms in the major chain, i. e. whether it is a pentose or hexose.

Recently Lindberg et al[30] have developed a method for the unambiguous determination of partially methylated sugars using combined gas-liquid chromatography - mass spectrometry. Partially methylated alditol acetates, obtained from methylated sugars by borohydride reduction followed by acetylation, are readily separable by gas chromatography on an ECNSS-M column.[25] The mass spectrum is taken at the beginning, maximum and end of each main peak obtained by g.l. c. From the fragmentation pattern the type of substitution is readily determined and from the retention time the parent sugar can be determined providing reference samples are available.

Mass spectrometry is useful since for each substitution pattern a unique spectrum is obtained, which is, unfortunately, not unique to the parent sugar; the mass spectrum of the 2, 4 - di - o - methylgalactose derivative is identical with that of 2, 4 - di - o - methylglucose but different from the 3, 4 - derivatives. Hence the parent sugar is determined from the T values (g. l. c.) and the substitution pattern from the mass spectrum. Due to the inherent symmetry of alditols, information will sometimes be lost when alditol acetates of methylated sugars are formed. For example, the alditols derived from 2, 3 - and 3, 4 - di - o - methylpentoses will give identical mass spectra, as will those derived from 2, 4 - and 3, 5 - di - o - methylhexoses. Reduction with borodeuteride eliminates this disadvantage.

The mass spectra of alditol acetates contain two types of fragments - primary and secondary. The former result from fission between two carbon atoms in the alditol chain (either of the two fragments formed could carry the positive charge), and the latter are formed from the primary fragments by elimination of a small moeity such as acetic acid or ketene. The differences in the mass spectra of different partially methylated alditol acetates are as a result of the primary fragmentation; fission of the C chain occurs preferably between two adjacent methoxyl groups or, at least, next to a methoxyl group rather than between two acetoxyl groups since the positive charge is stabilized by a methoxyl group.

For the successful, unambiguous investigation of the methylation products of a heteropolysaccharide it is necessary to know the sugar composition of the original polysaccharide, T values of possible alternative derivatives and to have the mass spectrum of a methylated alditol acetate having the same substitution pattern. Use of this method

has been made in the investigation of cell-wall lipopolysaccharides[31] where only small quantities of material are available.

This method of investigating methylated products can be directly utilized in the elucidation of structure of oligosaccharides. After methylation, hydrolysis followed by reduction and acetylation will yield two or more products which can be readily identified by their mass spectra and T values. Alternatively, the mass spectrum can be taken of the methylated disaccharide hexitol.[32] The occurrence of a 1, 6 - or 1, 2 - linked disaccharide hexitol can be determined by the presence of four carbon chain fragments having m/e 177 and 145 (177 - MeOH) which cannot be formed from hexitols with 1, 3 - and 1, 4 - linked structures. Corresponding ions (m/e 178 and 146) will be obtained in the mass spectra of the deuterated derivative of a 1, 6 - linked disaccharide, but not in that of a disaccharide hexitol possessing a 1, 2 - linkage. Similarly, the ion at m/e 133 arising through primary cleavage of the bond between C_3 and C_4 in the hexitol moeity differentiates 1, 3 - linked disaccharides (no deuterium effect) from 1, 4 - linked disaccharides. Hence the glycosidic linkage of any disaccharide hexitol can be assigned on the basis of the two ions, m/e 133 and 177.

Another approach in the investigation of oligosaccharides by g. l. c. - mass spectrometry is to incorporate an aromatic group into the molecule.[33] This stabilises the molecular ion and directs the fragmentation. From the mass spectrum it is possible to determine the sequence of masses of the constituent monosaccharides and the position of glycosidic linkages.

1. 4 Zone Electrophoresis (Ionophoresis)

Zone electrophoresis is another form of chromatography. In this case mobility depends upon the charge on the molecule, and the substance moves along the paper as the result of application of a high voltage gradient between the ends of the pherogram. Zone electrophoresis is thus directly applicable to those groups of carbohydrates which carry a net charge e. g. glycuronic acids, amino-sugars, sugar sulphates. However, the technique has been extended to sugars or their derivatives which are electrically neutral, since they can acquire a net charge by means of complex formation in certain electrolyte solutions e. g. sodium borate (pH9-10), sodium arsenite (pH9. 6), basic lead acetate (pH 6. 8) and sodium molybdate (pH 5). Location of the material on the supporting matrix is accomplished by making use of specific chemical reactions (e. g. reducing power) or physical properties (e. g. UV absorption) of the migrating substances. The chief advantage of zone electrophoresis is speed since a pherogram is run in a fraction of the time required for a paper chromatogram - usually $\frac{1}{2}$ - 2 h suffices. Zone electrophoresis and elution chromatography are useful as complementary techniques since quite different factors control zone electrophoretic (M values) and chromatographic mobilities (R values).

With neutral sugars the M values depend chiefly on the extent the sugar can complex with the buffer, e. g. borate, since this determines the residual charge. Böeseken[34] inferred that for cyclic carbohydrates only vicinal cis-hydroxyl groups can form a complex with borate ions in aqueous boric acid, and hence substances which are difficult to separate by paper chromatography may readily be separated by zone electrophoresis e. g. Foster[35] found that this applied to mixtures of

2, 4 - and 3, 4 - di - o - methyl - L - rhamnose since only the latter can form a complex with borate ions. Conversely, substances which are readily separable by paper chromatography, e. g. D-xylose and D-glucose, have very similar M values.

These examples illustrate how useful a combination of the two techniques can be, both in checking homogeneity of individual compounds and identity. A suitable situation would be checking fractions obtained after separating a mixture of carbohydrates using paper or column chromatography.

Zone electrophoresis has limited usefulness in the investigation of methylation products since many highly methylated carbohydrate derivatives are unable to complex with borate ions. However, in the di- and mono-o-methyl series there is generally a much wider variation in M values than in R values.

Foster[36] has found that the M values of glucose disaccharides depend on the position of the glycosidic linkage. Here the presence of a second sugar moeity in the molecule alters the complexing ability.

Carbohydrates of different molecular sizes e. g. pentoses and hexoses, may have identical M values in borate buffer. Hence this system cannot be used for determining molecular size. However, methods have been developed whereby the molecular weight of an aldose can be ascertained. One such is the method of Stacey et al.[37] The aldose is converted into the N-benzyl glycosylamine on the pherogram by reaction with benzylamine. The glycosylamine migrates as the glycosyl ammonium ion in a formic acid - sodium formate buffer (pH 1. 8). The mobility of the N-benzyl glycosylammonium ions under these conditions is inversely proportional to the molecular weight of the ions, and

independent of the stereochemistry of the sugar and of any glycosidic linkages present.

Fibre-glass sheets have been used in certain cases in place of the standard cellulose pherogram. This is particularly useful where quantitative work is being done since there is no danger of any carbohydrate contamination, or where cellulose interferes with the method used to detect the zones of migrating material. In general, M values are almost identical with those on paper.

Preparative zone electrophoresis is very tedious. Instead a column technique has been developed in which a standard charcoal - Celite column is impregnated with borate buffer and the eluant contains this buffer at the same pH. Saccharides which complex strongly with borate ions are eluted more rapidly from the column than would be the case in the absence of borate.[38]

1. 5 Partial Solvolysis

Partial solvolysis is the technique which provides the most information about the fine structure of a polysaccharide since the results are completely unambiguous. The polysaccharide is solvolysed partially by one of several methods to yield di-, tri- and oligo-saccharides. Complete elucidation of these smaller molecules enables one to determine the order of monosaccharides and their linkage.

It is possible to obtain information about the position of the sulphate groups in a sulphated polymer by means of partial hydrolysis since the rate of hydrolysis of sulphate esters with aqueous acid is similar to that

of glycosidic linkages.[39] Certain sulphated mono- and oligosaccharides will be obtained in addition to the neutral products. This method of partial acidic hydrolysis was used by Turvey and Rees[40] to determine the position of sulphate residues in porphyran. They calculated that after 5h hydrolysis with 0.29M sulphuric acid at 50° about 27% of the monosaccharide sulphates in the original polysaccharide would be present in the hydrolysate. The only sulphated product isolated was L-galactose-6-sulphate; in porphyran the number of sulphate ester groups exceeds the number of L-galactose residues, hence not all the sulphate can occur as L-galactose-6-sulphate, it must be assumed that the other ester sulphates present are more acid labile. If the polymer contains uronic acids the conditions needed for partial hydrolysis are more vigorous with the result that all the sulphate groups will be removed.

The most common methods of partially cleaving a polysaccharide are acid or enzymic hydrolysis and acetolysis; each method can yield a different series of oligosaccharides. All glycosidic bonds do not show the same susceptibility to attack by chemical reagents. With mineral acid, attack occurs more rapidly at furanosidic than pyranosidic linkages, (1→4) - linkages are more resistant to attack than (1→3) - linkages, α -glycosidic bonds are usually more labile than β -, and pentaglycans in the pyranose form are more readily hydrolysed than pyranoside hexoglycans.[41] Increased resistance to acid hydrolysis is conferred on polysaccharides by the presence of uronic acid groups and by amino sugars.

In polysaccharides containing both normal and easily hydrolysable linkages, it is possible to cleave practically all the latter with little

hydrolysis of the former, although in practice some cleavage of the more resistant linkage is inevitable. Investigation both of the easily hydrolysed oligosaccharides and of the non-hydrolysed polysaccharide can yield valuable information. Thus Jones,[42] by the isolation of 3 - o - β - L - arabinopyranosyl - L - arabinose after a mild hydrolysis of larch arabinogalactan, showed that the polysaccharide must contain residues corresponding to this disaccharide attached to the rest of the molecule by furanosidic linkages.

With acid hydrolysis care should be taken since the reaction is reversible, and there is, thus, the possibility that, if the reaction is carried out using concentrated acid, oligosaccharides may be formed which are not characteristic of the hydrolysed polysaccharide. However, in dilute solutions ($< 1\%$) the reverse reaction is unimportant, and only insignificant amounts of oligosaccharides are formed.[43]

Acetolysis is carried out by treating a polysaccharide with a reagent such as acetic anhydride - sulphuric acid which causes cleavage of an O-R bond with concomitant formation of an acetic ester. Other systems that have been used are[44] acetic anhydride - perchloric acid, acetic anhydride - zinc (II) chloride and trifluoroacetic anhydride - acetic acid. All of these reagents give rise to the acetylium ion $[CH_3CO]^+$ which is the attacking species. Cleavage of sulphate esters also occurs. The resultant oligosaccharide peracetates may readily be deacetylated to yield the oligosaccharide.

Acetolysis is complementary to acid hydrolysis. For example, in the former 1, 6- linkages are the most susceptible to attack; in the latter they are the least easily ruptured. Presumably the 1, 6- linkage is split preferentially by acetolysis because of the greater ease of approach of the acetylium ion to that site than to a secondary-secondary linkage.[45] Hence, the oligosaccharide fragments obtained from a

complex polysaccharide by each method are not identical.

When pure enzymes are available enzymic hydrolysis is very useful since, in addition to information obtained by investigating the resultant oligosaccharides, the configuration and linkage of the ruptured bond is also known. For example, Araki[46] isolated an enzyme from agar digesting bacteria which yields neo-agarobiose (o-3, 6-anhydro-α-L-galactopyranosyl-(1→3) D-galactose) from agar. A κ-carrageenase[47] isolated from the cell-free culture medium of Pseudomonas carrageenovora degrades κ-carrageenan to reducing oligosaccharides, enzymic hydrolysis occurring at the β(1→4)-linkage.[48] The disaccharide obtained was shown to be neo-carrabiose-4-sulphate. An extracellular enzyme from a Cytophaga species of the Myxobacterium specifically cleaves the linkage between 3-linked β-D-galactopyranose and 4-linked 3, 6-anhydro-α-L-galactopyranose units.[49] The rate of hydrolysis is decreased if the D-galactose is present as the 6-o-methyl derivative or if the L-galactose occurs as the 6-sulphate.

An elegant method of improving the yield of oligosaccharides was devised by Painter[50] who carried out the enzymic hydrolysis in dialysis tubes, thus obtaining continuous removal of the low molecular weight products. Partial hydrolysis is, in general, a relatively non-specific process, causing cleavage of a wide range of bonds with the production of many different oligosaccharides. However, it is sometimes possible to get specific cleavage of a polysaccharide into oligosaccharides; this selectivity is governed by the structure of the polysaccharide.

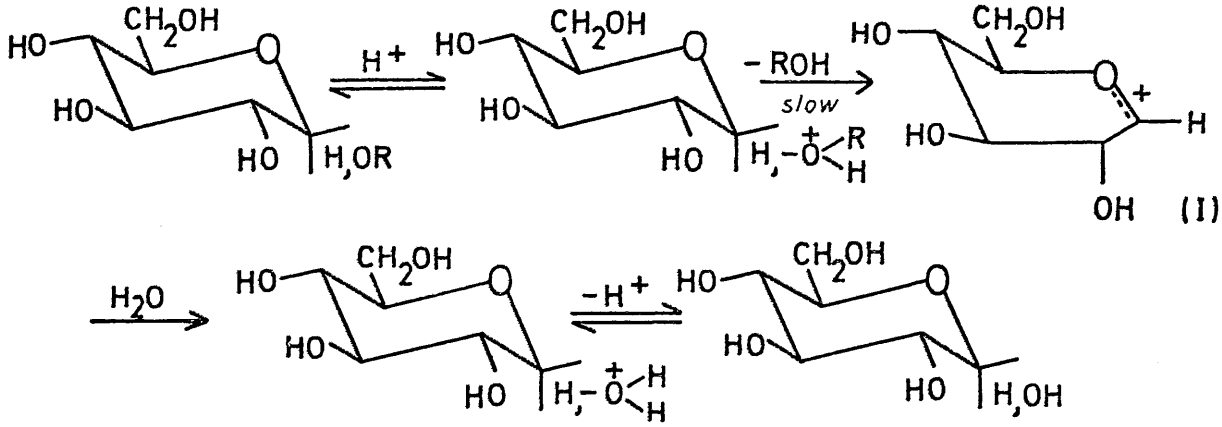
3, 6-Anhydrogalactose is very unstable to acid hydrolysis being decomposed to 5-hydroxymethyl-2-furaldehyde which is further degraded to levulinic acid and formic acid.[51] Treatment with methanolic hydrogen chloride results in the formation of 3, 6-anhydrogalactose dimethyl acetal. When 3, 6-anhydrogalactose occurs

in a polysaccharide mild methanolysis results in cleavage of the glycosidic bond, and formation of the dimethyl acetal derivative.[52] This reaction was utilized to show the repeating structure of κ-carrageenan.[53] κ-Carrageenan was known to contain a high proportion of carrabiose (3, 6- anhydro - 4 -o - β - D - galactopyranosyl - D - galactose) units which suggests a structure containing an alternating chain of galactose and 3, 6 - anhydrogalactose units. Mild methanolysis of the polysaccharide resulted in an 88% yield of carrabiose indicating the proportion of 3, 6- anhydrogalactose that exists in the form of disaccharide repeating units. Methylation studies on κ -carrageenan indicate the presence of 1, 4- linked galactose -2, 6 - disulphate in addition to 1, 3- linked galactose - 4 - sulphate and some 3, 6 - anhydrogalactose - 2 - sulphate. Alkali treatment of κ-carrageenan increased the 3, 6 - anhydrogalactose content by 6%. The modified polysaccharide had a carrabiose content of 99%; this increase can only be explained if the galactose - 6 - sulphate units replace 3, 6 - anhydro-galactose units in the repeating chain.

Clingman and Nunn[54] partially methanolysed Hypnea specifera to obtain 3, 6 - anhydrogalactose dimethyl acetal and a disaccharide dimethyl acetal, the latter being characterised by reduction to the glycitol after hydrolysis with dilute oxalic acid.

In addition to methanolysis, mercaptolysis[55] has been used to isolate 3, 6- anhydrogalactose. The polysaccharide is treated with ethylmercaptan in the presence of concentrated hydrochloric acid at low temperature. The acid hydrolyses the polysaccharide and the resulting reducing sugars are simultaneously converted into their respective diethylmercaptals.

In polysaccharides containing uronic acids aldobiouronic acids are readily isolated because of the strong resistance of the uronosyl linkage to acid hydrolysis. The generally accepted mechanism for acid hydrolysis of glycosides[56] involves a cyclic carbonium-oxonium ion (I) as an intermediate and the stability of this ion will



influence the rate of heterolysis of the glycosidic bond. A carboxyl group at C_6 would, as a result of its electron withdrawing property, destabilise (I) and, thus, render the glycoside more stable to acid. McCleary et al[57] have attempted to devise methods for the cleavage of polysaccharide chains containing only uronic acid residues, by elimination yielding 4, 5-unsaturated units. 2-Hydroxyethyl alginate, which appears to consist entirely of 1, 4 - linked uronic acid derivatives was chemically dehydrated with 2, 2 - dimethoxypropane and then treated with dry methanolic sodium methoxide for 1h. A 30% yield of di- and oligosaccharides was obtained. These showed u. v. and i. r. bands characteristic of α,β-unsaturated esters. Evidence obtained from reduction and ozonolysis experiments and treatment with acid confirmed that the fragmentation was by elimination. The reaction does not stop with the 4, 5- unsaturated compounds, degradation occurs if the reaction is continued for a longer period of time and, in addition to the oligosaccharides mentioned, 50% of the starting material was insoluble in the reaction mixture and stable to further reaction.

Toluene-p-sulphonates stabilise glycosidic linkages to acid hydrolysis when they are present at C_2.[58] Presumably the carbonium ion at C_1 is destabilised by the electron withdrawing property of the tosyl group. Tosyl groups at C_6 also stabilise the glycoside to a useful extent.[59] This is especially useful for polysaccharides such as dextran and pectic arabinan in which unsubstituted primary hydroxyl groups occur only at the end residues. Selective tosylation allows the terminal linkages to be characterised, giving useful information about fine structure.

Partial hydrolysis has only come into widespread use since the development of chromatographic techniques, as generally the hydrolysates are fairly complex mixtures. Probably the most selective method for the fractionation of oligosaccharides or their acetates is chromatography on columns of Fuller's earth or calcium (or magnesium) acid silicate, a procedure which was developed by Wolfrom et al.[60] Chromatography on charcoal-Celite columns[61] is another valuable method since the effectiveness of the separation is not affected by small variations in the composition of the developer, by the degree of dilution of the sugar solution or by the presence of inorganic salts. An added advantage is that a mixture of substantially large quantities of sugars can be separated by the use of a single small column. As oligosaccharides have low R_F values, less use has been made of chromatography on cellulose columns.

In general, fractions eluted off columns do not contain only one component and further separation is necessary to obtain pure components. Possibly the simplest method is separation on paper chromatograms. The structures of the pure oligosaccharides can be determined by a combination of partial and total hydrolysis, reduction followed by hydrolysis, and methylation. Circular dichroism investigations on disaccharide molybdate complexes show that, as carbon atoms 1 to 3 are required for complex formation and C_4 is energetically or sterically hindered, only 6-o-pyranosyl

sugars can complex with molybdate. This leads to Cotton Effect bands above 200 nm. Thus, the circular dichroism spectrum of a disaccharide molybdate solution can readily differentiate 1, 6 - linked disaccharides from other types.[62]

1. 6 Nuclear Magnetic Resonance

The chief application of nuclear magnetic resonance (n. m. r.) to the field of polysaccharides has been in the determination of the configuration of glycosidic linkages in oligosaccharides. This supersedes the earlier method of calculation based on Hudson's rules.[63]

It was found[64] that the 60 MHz n. m. r. spectra of glycosides of glucose and galactose showed three distinct types of proton. The anomeric proton on C_1 appears at low applied magnetic field because the carbon atom to which it is attached bears two electron withdrawing oxygen atoms. The protons attached directly to C_2 - C_6 resonate in the region 5. 67-6. 67 τ and the spectrum is characteristic for the particular sugar moiety. The hydroxyl protons form an exchanging pool with the residual water in the deuterium oxide solution and have a single sharp resonance near 5. 25τ.

The actual position of the resonance of the anomeric proton depends on its configuration; for D-glucose a doublet at 4. 78 τ (J_{12} 3. 0 c. p. s.) was attributed to H^{α} , and a doublet at 5. 35 τ (J_{12} 7. 4 c. p. s.) to H^{β}. Integration of the signals assigned to the α-and β- anomers gives the percentage composition of the equilibrium mixture.

In a disaccharide, the glycosidically linked anomeric proton is of fixed configuration so in addition to peaks for H^{α} and H^{β}, a third, more intense, C_1 proton signal will be obtained. The position of this signal and the magnitude of the coupling constant gives the configuration of

the disaccharide. The anomeric proton of cellobiose resonates at 5.50 τ (J_{12} 7.4 c.p.s.) confirming the linkage to be β-, while maltose has a resonance for the α-linkage at 4.62 τ (J_{12} 3.4 c.p.s.).

Use has been made of the above values in the structural elucidation of the gum Araucaria bidwillii.[65] The proton magnetic resonance spectrum of a disaccharide alditol in deuterium oxide showed a doublet (5.39 τ, J 7.3 c.p.s.) attributable to the anomeric proton of the aldosyl moiety, indicating the configuration of the glycosidic linkage to be β-. A disadvantage of this technique is that a relatively large amount of material is necessary for n.m.r. spectroscopy.

N.m.r. has been used to identify the positions of substitution in partially or fully methylated D-galactopyranose derivatives.[66] The derived methyl glycosides are perdeuteromethylated, and the methoxyl chemical shifts in benzene compared with the methoxyl proton signals of 2,3,4,6-tetra-o-methyl α- and β-D-galactopyranosides at 100 MHz. It was found that the position of the signal for a particular proton depended on the solution concentration and solvent. In certain cases, the responses of two protons were inverted.

Mannans and galactomannans from yeasts have been examined by n.m.r. in deuterium oxide. It was found[67] that a complex signal due to the H_1 protons occurred at τ 4.0 - 5.0, the actual value of chemical shift depending on (i) the structure of the parent anhydrohexose, (ii) the position and structure of substituent(s), (iii) the structure of the aglycone and (iv) the position(s) of substitution in the aglycone. In fact, similarities in this region of the n.m.r. spectrum reflect similarities in chemical structure and mannans giving n.m.r. spectra with a lesser degree of similarity have markedly different chemical structures. The galactomannan

of Torulopsis gropengiesseri and the mannan of Saccharomyces rouxii give H_1 n. m. r. spectra with only slight differences.[68] Methylation fragmentation patterns, acetolysis and enzymolysis show that each polysaccharide contains an α-1, 6-linked D-mannopyranose main chain substituted in position-2. T. gropengiesseri has its main chain substituted by o-galactopyranosyl-o-D-mannopyranosyl-(1→2)-α-D-mannopyranose, while the S. rouxii side chains are 2-o-α-D-mannopyranosyl-D-mannopyranose.

1. 7 Oxidation Methods

Cleavage of α-glycols with periodate or lead tetraacetate has been used in a variety of ways for structural elucidation of polysaccharides. The consumption of oxidant by disaccharides[69] and the formation of formic acid (carbon dioxide) depends on the type of substitution of the reducing residue. These values can be used for distinguishing between alternative structures.

In polysaccharides, the amount of information obtained from periodate oxidation studies depends on the polymer, generally it is only significant for highly branched polysaccharides. Haq and Percival carried out a Smith Degradation,[70] oxidation with periodate followed by reduction and mild hydrolysis, on the highly branched sulphated polysaccharide obtained from Ulva lactuca.[71] Investigation of the hydrolysis products gave further insight into the detailed molecular structure.

Another modification of periodate oxidation is the Barry Degradation.[72]

After oxidation with periodate, the polyaldehyde is treated with phenylhydrazine and acetic acid. This results in removal of the oxidised residues as phenylosazones. This reaction can be used to bring about a regulated degradation of 1, 3- linked polysaccharides. Periodate only oxidises the non-reducing terminal sugar unit. Treatment with phenylhydrazine enables the polymer to be recovered shorter by one unit. The new non-reducing end is now susceptible to attack by periodate, and thus sugar units can be eliminated one by one from the non-reducing end of the polysaccharide chain. This method is useful for polysaccharides containing other glycosidic linkages, as degradation can result in a series of oligosaccharides of shorter chain length. If the polysaccharide contains a backbone resistant to oxidation this can be recovered from the reaction mixture. Repeated Barry degradations can be useful since removal of oxidised residues may result in the formation of new sites for attack by periodate.

Sometimes it is desirable to degrade periodate oxidised polysaccharides so that unattacked residues are split off as free reducing sugars. This can be achieved by a graded hydrolysis of the polyaldehyde with sulphurous acid.[73]

2. EXPERIMENTAL

Concentration of solutions was carried out at 40° under reduced pressure, and specific rotations were measured in water unless otherwise stated. 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) butyl alchohol-pyridine-water (9:2:2), (c) butyl alcohol-ethyl alcohol-water (40:11:9), (d) ethyl acetate-pyridine-water (8:2:1) and (e) ethyl acetate-pyridine-water (10:4:3). Sprays (i), (ii), (iii) and (iv) were, respectively, 2% p-anisidine hydrochloride in butyl alcohol containing 5% water;[74] 2% p-anisidine hydrochloride in butyl alcohol containing 5% 0.1M hydrochloric acid; equal parts of 5% triphenyltetrazolium chloride in methanol and IM methanolic sodium hydroxide, mixed just before use; 20% sulphuric acid in ethanol. Paper electrophoresis was performed on Whatman No. 1 paper with 0.4M borate buffer (pH 10) at 50 mA (solvent f) and 0.1M pyridine-acetic acid buffer (pH 6.5) at 20 mA (solvent g). R_{Gal} and M_G values refer to rates of movement of sugars relative to galactose on paper chromatograms and pherograms, respectively. Thin layer chromatography (t.l.c.) was carried out on glass plates coated with Silica Gel G (Merck) containing calcium sulphate as binder, using methyl ethyl ketone-water (85:7) as eluent. R_{TMG} values of methylated sugars refer to the rates of travel relative to that of 2,3,4,6-tetra-o-methyl-D-galactose. Sulphate determinations were carried out using the 4'-chlorobiphenyl-4-ylamine method.[75,76] The degree of polymerisation (D.P.) of oligosaccharides was determined by the phenol-sulphuric acid method.[77] Infrared spectra were recorded on a Beckman IR-8 spectrophotometer. Gas-liquid chromatography (g.l.c.) was carried out using a Beckman GC 4

chromatograph equipped with dual flame-ionization detectors and using nitrogen as carrier gas. Separations of methyl glycosides were made on columns containing (1) 15% by weight butan -1,4- diol succinate polyester on acid-washed Celite (80-100 mesh) at 175° and (2) 5% w/w neopentylglycol adipate on Chromosorb W (80-100 mesh; acid-washed) at 160°. G.l.c. of the alditol acetates was carried out using 20% Apiezon M on Chromosorb W (80-100 mesh; acid-washed and treated with dimethylchlorosilane) at 175°. Retention times T and T_E are relative to that of methyl 2,3,4,6-tetra-o-methyl - β -D-glucopyranoside and erythritol tetraacetate respectively.

2.1 Isolation and purification of polysaccharide

Steam was passed into wet Pachymenia carnosa (2 kg) in water with constant stirring, the pH being kept at 5-6 by the addition of glacial acetic acid when necessary. After the weed had disintegrated, steam was passed through the mixture for a further 0.5 h. The resulting slurry was fed into a basket centrifuge and the centrifugate precipitated in ethanol (5 vols.). The product was washed with ethanol, and finally with ether to give 203 g (10% yield on wet-weight basis) of crude, off-white, fibrous polymer.

Further purification of the polysaccharide for analysis was effected by repeated (4 times) dissolution in water, centrifugation of the solution and precipitation in ethanol (5 vols.). This yielded a white, fibrous polymer having (on material dried under high vacuum over P_2O_5 at 60° for 24 h) $[\alpha]_D^{20}$ + 73° (c 0.52); OMe, 2.2; N, 0.3; 3,6-anhydro-galactose,[78] 1.4; SO_4^{2-}, 30.3; $NaSO_3^-$, 32.5%; ν_{max} 1240 and 825 cm^{-1}

(KBr disc); equivalent (from SO_4^{2-} determination), 317.

Chromatographic examination of a neutralized acid hydrolysate (solvents a-d) revealed the presence of galactose (major; yellow-brown), 2-o-methylgalactose (yellow, goes pink), R_{Gal} 2.72 (solvent d), 4-o-methylgalactose (minor; yellow), R_{Gal} 1.77 (solvent d), 6-o-methylgalactose (brown), R_{Gal} 2.39 (solvent d), and xylose (trace; red) (spray i).

2. 2 Hydrolysis of polysaccharide and separation of the component sugars

Polysaccharide (8.6g) and sulphuric acid(0.38M; 100 ml) were heated for 16 h on a boiling-water bath. After neutralization ($BaCO_3$), centrifugation and evaporation to dryness, the residue was extracted with aqueous methanol. Evaporation of this solution gave a syrup (3.19g), which, in the minimum amount of water, was applied to a charcoal-Celite column (1:1w/w; 4.5 x 35 cm). Initially the column was eluted with water, and then by 0.5, 2 and 5% aqueous ethanol. Fractions (ca 15 ml) were collected and sorted by paper chromatography using solvents (a) and (b).

Fraction I. The syrup (1.73g), which was shown to contain galactose and a trace of xylose, was crystallised from aqueous ethanol. After further crystallization (aqueous ethanol) the sugar had, $[\alpha]_D^{23}$ + 120° (3 min)→+ 72° (c 0.50), m.p. and mixed m.p. 161-162° with an authentic sample of D-galactose. The sugar (50 mg) in nitric acid - water (1:1; 1 ml) was heated on a water bath at 80° for 2 h. The mucic acid which crystallised on cooling had, after recrystallization from water, m.p. and mixed m.p. 212-213° with authentic mucic acid.[79]

Fraction II. The syrup (0.175g) was shown by paper chromatography to contain galactose and 2-o-methylgalactose (major sugar).

Fraction III. The syrup (0.410g) contained 2-o-methylgalactose (major) and 4-o-methylgalactose (paper chromatography). The 2-o-methyl ether crystallised from methanol and had $[\alpha]_D^{20}$ + 54° (3 min) → + 84° (c 0.5), m.p. and mixed m.p. 148-149° with authentic 2-o-methyl-D-galactose. The mother liquor was concentrated and separated on several sheets of paper (solvent d). The areas corresponding to the 4-o-methyl ether were extracted with methanol, filtered and evaporated to a syrup (10 mg) which had $[\alpha]_D^{22}$ -9° (c 0.77) on a sample dried at 60°/0.1 torr. The sugar (8 mg), freshly distilled aniline (16 mg) and a drop of acetic acid in ethanol (0.5 ml) were refluxed for 4 h. The 'anilide' after recrystallization from ethanol had m.p. 166-168°. A reported value[80] is m.p.167-168° for 4-o-methyl-L-galactose and 168°[81] for 4-o-methyl-D-galactose.

Fraction IV., a syrup (0.225 g) was (paper chromatography) a mixture of 2-o-methylgalactose (major) and 6-o-methylgalactose.

Fraction V. The syrup (0.350 g) contained (paper chromatography) 6-o-methylgalactose and a trace of 2-o-methyl-D-galactose. The 6-o-methylgalactose crystallised as plates from ethanol-ethyl acetate and had $[\alpha]_D^{19}$ + 138° (2 min) → + 80° (c 0.65), m.p. 119-121° and mixed m.p. 116-117° with authentic 6-o-methyl-D-galactose.

2.3 Quantitative estimation of the polysaccharide components

The component sugars of the polysaccharide were estimated quantitatively by gas chromatographic examination of their alditol acetates based on the method of Bowker and Turvey.[82] Dry polysaccharide (105.05 mg) and erythritol (2.12 mg) were dissolved in 0.5M sulphuric acid (3 ml) and the solution heated at 100° for 3h. The cooled solution was neutralized ($BaCO_3$) and the barium salts removed

by centrifugation. Sodium borohydride (20 mg) was added to the supernatant solution, which was allowed to stand at room temperature for 18 h and then neutralized with 0.5M sulphuric acid. The resultant solution was evaporated and dried at 30°, 0.1 torr. Acetylation was carried out by adding acetylating mixture (2ml; 50:1 v/v acetic anhydride: sulphuric acid) to the dried residue, and heating the stoppered container at 80° for 6 h. When cool, water (2 ml) was added and the mixture cautiously neutralized to pH 5 with sodium hydrogen carbonate, and then extracted with chloroform (2x20 ml). The combined chloroform extracts were dried ($MgSO_4$), concentrated to 2 ml, and injected (1 microlitre samples) onto the gas chromatograph.

In order to quantitatively estimate the component sugars, standard curves were prepared for all the components by reducing and acetylating samples of the sugars (ca 30 mg) and erythritol (ca 4 mg). The molar response for each component was determined, and the concentration of the given sugar in the polysaccharide was estimated from the peak area of its glycitol acetate relative to that of erythritol tetraacetate.

2.4 Alkali treatment of the polysaccharide

To polysaccharide (1.0 g) in water (75 ml) was added sodium borohydride (0.2g) and the solution set aside at room temperature for 48 h, with occasional shaking. Sodium hydroxide (7g) and sodium borohydride (0.8g) were then added and the mixture heated to, and maintained at, 80° (± 2°). After 4 h a second aliquot of sodium borohydride (0.8g) was added. After a further 3 h the solution was cooled and made slightly acid with hydrochloric acid, dialysed and concentrated. The polymer was isolated by freeze-drying to a white

foam (877 mg) having $[\alpha]_D^{19}$ + 76° (c 1.0), (Found: SO_4^{2-}, 30.6; 3,6- anhydrogalactose,[78] 2.35%). Paper chromatography of an acid hydrolysate showed the presence of only the sugars found in the native polymer, viz. galactose, 2-o-methylgalactose, 4-o-methylgalactose, 6-o-methylgalactose and xylose (trace). The chromatogram was slightly streaked due to the presence of 3,6-anhydrogalactose.

2.5 Treatment of polysaccharide with sodium methoxide

A modified Percival and Wold[83] procedure was utilized. To polysaccharide (1.0g) dissolved in water (50 ml) was added sodium borohydride (0.4g) in two portions over 48 h. The solution was then dialysed, concentrated and the reduced polymer isolated by freeze-drying (807 mg). The polyalcohol (dried at 60°/0.01 torr over P_2O_5 for 48h) was added to a solution of sodium (3.0g) in absolute methanol (125ml), and the mixture refluxed for 24h under anhydrous conditions. The insoluble material was removed by filtration, dissolved in water (100 ml) and dialysed for 3 days against frequently changed distilled water. Concentration and freeze-drying yielded a white foam (528 mg). Paper chromatography of an acid hydrolysate (solvents a-d) showed the presence of galactose, 2-o-methylgalactose, 4-o-methylgalactose, 6-o-methylgalactose and xylose (trace). No spot with the mobility of a di-o-methylhexose could be detected.

2.6 Partial hydrolysis of the polysaccharide

In order to determine the optimum conditions for the formation of oligosaccharides, polysaccharide (0.75 g) in sulphuric acid(0.5 M; 15 ml)

was heated on a boiling-water bath. Aliquots (1 ml) were withdrawn at regular intervals, neutralized ($BaCO_3$), centrifuged, concentrated and subjected to paper chromatography (solvents a and d) for 16 h. The optimum time of hydrolysis for the production of the maximum amount of oligosaccharides was 2.5 h. Polysaccharide (20 g) was hydrolysed (0.5M sulphuric acid, 400 ml) for 2.5 h under the above conditions, neutralized ($BaCO_3$), centrifuged, and evaporated to dryness. The product (12.9g) in the minimum quantity of water was deionized by passage, first through a column (20 x 4 cm) of Amberlite IR-120 (H^+) resin and then through a column (21 x 5 cm) of Amberlite IRA 400 resin in the acetate form. In each case the columns were washed until free of carbohydrate. Evaporation of this solution yielded a white foam (7.1g). This neutral material was applied to a charcoal-Celite column (1:1 w/w; 65 x 5.4 cm), which was eluted with water and aqueous ethanol (2-30%) using the gradient technique. Fractions (ca 30 ml) were collected and sorted into 11 major fractions with the aid of paper chromatography.

Fraction I. The partially crystalline white foam (2.20g), eluted with water (0.7 l), was shown chromatographically to be predominantly galactose. It also contained traces of xylose and a sugar of R_{Gal} 2.7 (solvent d).

Fraction II. The syrup (553 mg), eluted with water (3.6 l), consisted of galactose, 2-o-methylgalactose and 4-o-methylgalactose (paper chromatography).

Fraction III. The syrup (458 mg), eluted with water (9.0 l), consisted of galactose (trace),4-o-methylgalactose, 2-o-methylgalactose (major component) and a sugar (yellow,turns red; spray i) of R_{Gal} 3.70 (solvent a), 4.35 (solvent d) which is not revealed with spray iii. In order

to isolate the fast moving sugar the syrup (235 mg) was separated on Whatman No. 1 paper (solvent d; 24 h). Extraction of the relevant portion of the papers with water-methanol (1:1) yielded a syrup (48 mg), $[\alpha]_D^{21}$ 0° (c 0.96). Demethylation[74] of the sugar (2 mg) with 48% hydrobromic acid (0.2 ml) on a boiling-water bath for 5 min followed by chromatography (solvents a and d) of the neutralized ($AgCO_3$) solution revealed the presence of a sugar with the mobility of galactose and one (yellow; spray i) which had R_{Gal} 1.57 (solvent a), 3.04 (solvent d). The sugar was unchanged on acid hydrolysis(0.5M H_2SO_4, 16 h at 100°) and on exhaustive methylation gave a product chromatographically identical to 2,4,6-tri-o-methylgalactose (solvent d).

Fraction IV. The syrup (363 mg) was eluted with 2% aqueous ethanol (7.2 l) and shown (paper chromatography) to consist predominantly of a sugar chromatographically indistinguishable from 6-o-methylgalactose. It also contained traces of sugars having R_{Gal} <1 (solvent a).

Fraction V. The syrup (464 mg) was eluted with 5% aqueous ethanol (4.5 l). Paper chromatography showed that the fraction was a single sugar having R_{Gal} 0.39 (solvent a), 0.27 (solvent d). It crystallized readily from aqueous methanol with m.p. 204-205°, $[\alpha]_D^{20}$ + 77° (4 min) → + 67° (c 0.52). Partial hydrolysis followed by paper chromatography revealed the presence of galactose and the original material, while complete hydrolysis gave galactose only. A portion[5] (5 mg) was dissolved in redistilled N,N- dimethylformamide (0.5 ml) and the solution cooled to 0°C. Redistilled methyl iodide (0.5 ml) and dry, freshly prepared silver oxide (0.5 g) were added and the mixture stirred vigorously in the dark in ice for 3 h, and then at room temperature for 24 h. The partially methylated oligosaccharide was chloroform extracted

from the silver salts. After a single treatment with Purdie's reagents,[4] t.l.c. of the product revealed (spray iv) the presence of 2 discrete spots, indicating that methylation was complete. Acid hydrolysis (0.5M sulphuric acid, 4 h) of the methyl glycosides revealed (t.l.c. and paper chromatography) only two spots having the mobilities of 2,3,4,6-tetra-o-methylgalactose (R_{TMG} 1.00) and 2,3,6-tri-o-methylgalactose (R_{TMG} 0.87). A sample of the methylated oligosaccharide was refluxed with 3% methanolic hydrogen chloride for 6 h and the derived methyl glycosides examined by g.l.c. (column 1). Peaks corresponding to 2,3,4,6-tetra-o-methylgalactose (T 1.80) and 2,3,6-tri-o-methylgalactose (T 3.17, 3.89, 4.20, 4.49) in the molar ratio 1.0:1.00 were observed. The infrared spectrum (KBr disc) of this oligosaccharide was identical with that of authentic 4-o-β-D-galactopyranosyl-D-galactose.[84]

Fraction VI. The syrup (136 mg) was eluted with 5% aqueous ethanol (4.5 l) and shown by paper chromatography to contain mainly 4-o-β-D-galactopyranosyl-D-galactose and a sugar with R_{Gal} 0.62 (solvent a). Separation of this fraction on Whatman No. 1 paper (solvent a, 72 h) followed by extraction of the appropriate portions of the papers with 50% aqueous methanol afforded a chromatographically pure syrup, $[\alpha]_D^{21}$ + 32° (c 0.50), R_{Gal} 0.62 (solvent a), 0.36 (solvent d); it gave a red spot with spray (iii) indicating a free hydroxyl group on C_2 of the reducing end of the molecule. Partial hydrolysis of the sugar followed by paper chromatography revealed the presence of galactose and 2-o-methylgalactose in addition to the original material, while total hydrolysis gave galactose and 2-o-methylgalactose only. To sugar (4 mg) dissolved in water (2 ml) was added sodium borohydride (6 mg) and the mixture allowed to stand overnight. The solution was then passed

through Amberlite IR - 120 (H^+) resin, evaporated and freed of borate by repeated distillation with methanol. Hydrolysis of the non-reducing syrup, followed by paper chromatography (solvent d; spray i) revealed the presence of 2-o-methylgalactose as the only reducing sugar. Methylation of the sugar (5 mg) as before gave a fully methylated oligosaccharide (t. l. c.; spray iv). A portion of this sample was methanolysed by refluxing it with 3% methanolic hydrogen chloride for 6 h and the derived glycosides examined by g. l. c. (column 1). Peaks characteristic of 2, 3, 4, 6-tetra-o-methylgalactose (T1. 83) and 2, 4, 6-tri-o-methylgalactose (T 3. 91, 4. 19) were observed. Paper chromatography of a hydrolysate of the methylated oligosaccharide revealed only 2, 4, 6-tri-o-methylgalactose and 2, 3, 4, 6-tetra-o-methylgalactose. No trace of a sugar with the mobility of 2, 3, 6-tri-o-methylgalactose was observed. The above results indicate that the oligosaccharide is 3-o-(2-o-methylgalactopyranosyl)-galactose.

Fraction VII. The syrup (134 mg) eluted with 7. 5% aqueous ethanol (1. 8 l) was found to be a mixture of five oligosaccharides. Separation of the fraction on Whatman No. 1 filter paper (solvent a, 60 h) followed by double extraction with methanol - water (1:1) yielded a chromatographically pure sample of the major sugar R_{Gal} 0. 96 (solvent a), 0. 76 (solvent d) (46 mg), which crystallised from methanol m. p. 213-214°, $[\alpha]_D^{18}$ + 86° (3 min) → + 62° (c 0. 49). Partial hydrolysis followed by paper chromatography revealed the presence of galactose and 2-o-methylgalactose in addition to the original sugar, and complete hydrolysis revealed the presence of approximately equal amounts of galactose and 2-o-methylgalactose. Reduction of the oligosaccharide with borohydride followed by paper chromatography of the acid

hydrolysate revealed the presence of galactose only (spray i). The sugar (5 mg) was methylated and methanolysed and the derived methyl glycosides examined by g. l. c. (column 1). Peaks corresponding to 2, 3, 4, 6-tetra-o-methylgalactose (T 1. 80) and 2, 3, 6-tri-o-methylgalactose (T 3. 19, 3. 98, 4. 21, 4. 48) in the molar ratio 1. 0:1. 10 were observed. This oligosaccharide is therefore 4-o- β -D-galactopyranosyl-2-o-methyl-D-galactose. The β-configuration is assumed from the optical rotation of the compound.

Fraction VIII. The syrup (472 mg), eluted with 10% aqueous ethanol (10. 5 1), was found (paper chromatography, solvent d) to consist predominantly of 4-o- β -D-galactopyranosyl-2-o-methyl-D-galactose and 4-o- β -(6-o-methyl-D-galactopyranosyl)-D-galactose. (See fraction IX). Traces of other oligosaccharides were also observed.

Fraction IX. The syrup (205mg) was eluted with 10% aqueous ethanol (4. 3 1). Paper chromatography (solvent a) revealed the presence of several saccharides. Separation on Whatman No. 1 filter paper (solvent a, 48 h) afforded the main component (51 mg), R_{Gal} 0. 96 (solvent a), 0. 68 (solvent d), $[\alpha]_D^{18}$ + 35° (c 0. 52). Partial hydrolysis of a sample yielded galactose, 6-o-methylgalactose and a small amount of the original sugar, while only galactose and 6-o-methylgalactose were obtained on total hydrolysis. On examination of the acid hydrolysate of a reduced sample by paper chromatography, only 6-o-methylgalactose was observed with spray (i). The sugar was methylated using the modified Kuhn procedure (5) followed by a treatment with Purdie's reagents, (4) and the derived methyl glycosides methanolysed using 3% methanolic hydrogen chloride and examined by g. l. c. on column 1. Peaks corresponding to 2, 3, 4, 6-tetra-o- methylgalactose (T 1. 83) and 2, 3, 6-tri-o-methylgalactose (T 3. 21, 3. 79, 4. 18, 4. 49) were observed in the molar ratio of 1. 0:1. 03. The

above results indicate that the oligosaccharide was 4-o- β -(6-o-methyl-D-galactopyranosyl)-D-galactose. The β- configuration is assumed from the oligosaccharide's optical rotation.

Fraction X. The syrup (243 mg) was eluted with 15% aqueous ethanol (7.5 l) and shown to contain several slow moving oligosaccharides (paper chromatography). This fraction was not further investigated.

Fraction XI. The syrup (253 mg) eluted with 20% aqueous ethanol (3.0 l) was found to contain traces of many oligosaccharides having $R_{Gal} < 1$ as well as two fast moving oligosaccharides. It was separated on Whatman No. 1 paper (solvent a, 24 h) and the relevant portions of the papers extracted with aqueous methanol, yielding the following two sub-fractions.

Fraction XIA. The chromatographically pure syrup (91 mg) R_{Gal} 1.84 (solvent a), 1.98 (solvent d), $[\alpha]_D^{21}$ + 54° (c 0.59) yielded 2-o-methylgalactose and 6-o-methylgalactose on partial acid hydrolysis. Reduction with borohydride followed by paper chromatography of the acid hydrolysate of the non-reducing syrup revealed (spray i) the presence of 6-o-methylgalactose. A portion of the oligosaccharide (5 mg) was methylated, methanolysed and the derived methyl glycosides examined by g.l.c. Only peaks corresponding to 2, 3, 4, 6-tetra-o-methylgalactose (T 1.89) and 2, 3, 6-tri-o-methylgalactose (T 3.27, 4.01, 4.25, 4.54) (column 1) in the molar ratio 1.0:1.13 were observed. The above evidence suggests that the oligosaccharide is 4-o- β -(6-o-methyl-D-galactopyranosyl)-2-o-methyl-D-galactose. The configuration is assumed to be β- from the optical rotation of the saccharide.

Fraction XIB. The syrup (4mg) had R_{Gal} 2.28 (solvent a), 2.87 (solvent d), $[\alpha]_D^{20}$ + 7° (c 0.88). Paper chromatographic examination of

a partial acid hydrolysate of the sugar revealed the presence of 2-o-methylgalactose and 6-o-methylgalactose only. Reduction of a portion of the saccharide (2 mg) with borohydride and paper chromatographic examination of an acid hydrolysate of this non-reducing syrup revealed 2-o-methylgalactose only (spray i). The above evidence suggests that the oligosaccharide is a 2-o-methylgalactopyranosyl-6-o-methylgalactose.

2. 7 Investigation of the acidic components of a partial hydrolysis of the polysaccharide

Polysaccharide (10g) was hydrolysed (0. 5M H_2SO_4; 200 ml) for 2. 5 h at 100°, and the hydrolysate applied to a column of Amberlite IRA 400 resin in the acetate form. Neutral material (6. 67g), which was discarded, was eluted from the column with water (1.0 l) and acidic material with 0. 5M sulphuric acid (2. 5 l). The acidic eluate was neutralised ($BaCO_3$), centrifuged, concentrated and deionized by passage through a column of Amberlite IR-120 (H^+) resin. Freeze-drying yielded the acidic product (1. 68g) which was neutralised with ammonia. Electrophoretic (solvent g; spray ii) and paper chromatographic (solvents a and e; spray i) examination of this material showed the presence of several acidic sugars. Separation of a portion (250mg) of this fraction in solvent e, followed by isolation and reseparation in solvent a, yielded a chromatographically (solvents e and a) and electrophoretically (solvent f) pure sugar.

The acidic sugar (14 mg) which had R_{Gal} 0. 56 (solvent e) M_G 1. 45 (solvent f), D. P. 1. 0, $[\alpha]_D^{20}$ + 54° (c 0. 22; calculated as the ammonium salt of a hexose monosulphate) was chromatographically and

electrophoretically identical to L-galactose-6-sulphate.[85] On hydrolysis (0.5M H_2SO_4, 4h, 100°) it gave galactose as the only reducing sugar. A portion of the sugar was methylated using the procedure of Haq and Percival.[5] Hydrolysis followed by paper chromatography (solvent d) showed the presence of 2,3,4-tri-o-methylgalactose; and a peak corresponding to methyl 2,3,4-tri-o-methylgalactosides (T 7.48, column 1) was obtained on investigation of a methanolysate by g.l.c. The galactose[86] to sulphate molar ratio was 1:6.25. The remaining material was deionized with Amberlite IR-120 (H^+) resin and the aqueous solution shaken with a 5% chloroform solution of tri-n-octylamine.[87] The aqueous solution, after washing with chloroform had a molar ratio of galactose[86] to sulphate of 1:2.8. Insufficient material remained for the sugar to be retreated with tri-n-octylamine until a constant galactose to sulphate ratio was obtained. The excess sulphate is attributed to contaminating ionic sulphate. The above evidence is consistent with the sugar being D-galactose-6-sulphate.

No other acidic compound could be obtained in a suitable condition for investigation.

2.8 Desulphation of polysaccharide[88]

Polysaccharide (1.0g) was shaken with 0.1M methanolic hydrogen chloride (75 ml) for 48 h at room temperature. The undissolved polysaccharide was removed by centrifugation, washed with dry methanol, dissolved in water (50 ml) and dialysed against frequently changed distilled water (3 days). Concentration and freeze-drying of the material in the dialysis sac gave a desulphated polysaccharide (583 mg, 79%)

(Found: SO_4^{2-}, 5.1%). The supernatant liquid was neutralized with silver carbonate, centrifuged and concentrated to a neutral syrup (382 mg).

The experimental conditions were varied slightly in three other trial desulphations.

Polysaccharide (1.0g) was shaken with methanolic hydrogen chloride (0.15M; 75 ml; 48 h) at room temperature to yield a desulphated polymer (554 mg, 78%) (Found: SO_4^{2-}, 1.7%) and supernatant syrup (565 mg). A second treatment under the above conditions gave a 77% yield of polysaccharide (Found: SO_4^{2-}, 0.8%).

Polymer (0.90g) was shaken with 0.1M methanolic hydrogen chloride (75 ml) for 72 h at room temperature to yield a desulphated polymer (429 mg, 66%) (Found: SO_4^{2-}, 3.1%) and supernatant syrup (411 mg).

Polymer (1.0g) was shaken with 0.15 M methanolic hydrogen chloride (75 ml) for 48 h at room temperature. The supernatant liquor was then removed by decantation, and the mixture shaken with a further aliquot of methanolic hydrogen chloride (0.15M; 75 ml) for 48h. The desulphated polymer (438 mg, 62%) (Found: SO_4^{2-}, 1.8%) was isolated by freeze-drying. Neutralisation of the combined supernatant liquors and concentration yielded a neutral syrup (484 mg).

Chromatographic investigation of neutralized acid hydrolysates (0.5M sulphuric acid; 16h; 100°) of the four non-reducing methanol-soluble syrups obtained above, revealed, in all cases, the presence of galactose, 2-o-methylgalactose, 4-o-methylgalactose, 6-o-methylgalactose as well as a sugar (trace) with R_{Gal} 3.88 (solvent a). Infrared examination (KBr disc) of the desulphated polymer (0.15 M methanolic hydrogen chloride for 48h; SO_4^{2-}, 1.7%) showed no absorbance at ν 820-830 cm^{-1}, and a very much reduced peak at ν 1240 cm^{-1} (fig. 1b).

Large scale desulphation was carried out by shaking polysaccharide (10.16g; dried over P_2O_5 for 48h, 0.1 torr, 60°) with methanolic hydrogen chloride (0.15 M, 750 ml) for 48 h at room temperature, and the product isolated by freeze-drying after dialysis. After three such treatments the product (3.74 g; 53%) had SO_4^{2-}, 0.9%.

2.9 Methylation of desulphated polysaccharide

To polysaccharide (4.0g; SO_4^{2-}, 0.9%) dissolved in dimethyl sulphoxide (100 ml) was added powdered sodium hydroxide (30g) and dimethyl sulphate (15 ml) over a period of 5 h, with constant stirring.[89] After stirring for a further 20 h the residual dimethyl sulphate was decomposed by the addition of excess ammonia. Water (100 ml) was added and the mixture dialysed against distilled water for 5 days. The partially methylated product (3.98g) was isolated by freeze-drying.

After two further treatments with the above reagents the dialysate was concentrated (to 100 ml) and repeatedly extracted with chloroform (8 x 60 ml). Evaporation of the combined chloroform extracts yielded a clear yellow gum (2.66g) (Fraction A; Found: OMe, 43.4%), the infrared spectrum ($CHCl_3$ solution) of which showed a very small hydroxyl peak. Further extraction with chloroform (5 x 60 ml) yielded a white syrup (0.64g) (Fraction B). The chloroform-insoluble polymer (0.90g; Fraction C) was isolated by freeze-drying. Fractions B and C were not further investigated.

Fraction A (2.66g) in methyl iodide (65 ml) was treated with freshly prepared silver oxide (22g) in portions while stirring under reflux for 8 h.[4] The mixture was filtered and the silver salts washed with

boiling chloroform. Concentration of the combined filtrates gave the methylated product (2.30 g; Found: OMe, 43.8%) (Theoretical for fully methylated hexose with 1% SO_4^{2-}, 44.2%). Further treatments with Purdie's reagents[4] failed to increase the methoxyl content.

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

Methylated polysaccharide (2.30g; OMe, 43.8%) was hydrolysed with aqueous formic acid[14] (90% v/v; 60 ml) for 1h at 100°. The formic acid was removed by concentration under reduced pressure, the syrup dissolved in sulphuric acid (0.25M, 100 ml) and the resulting solution kept at 100° for 16 h. Neutralization ($BaCO_3$), centrifugation and concentration yielded a yellow syrup which was applied to a charcoal-Celite column (1:1 w/w; 32 x 4 cm). The column was eluted with aqueous methyl ethyl ketone using the gradient technique (0→4% over 10 l). Fractions (ca 25 ml) were collected and analysed by paper chromatography (solvent d). Like fractions were combined to yield the following main fractions.

Fraction 1. The syrup (7mg) was shown to be a mixture of di-o-methylgalactoses (paper chromatography).

Fraction 2. The syrup (402mg) was shown by paper chromatography to contain several di-o-methyl sugars and 2,3,4-tri-o-methylgalactose (trace) in addition to 2,4,6-tri-o-methylgalactose. Separation of a portion (25 mg) of the fraction afforded chromatographically pure 2,4,6-tri-o-methylgalactose (14 mg) and a di-o-methyl fraction (9mg) containing sugars (solvent d) with the mobilities of 2,4-di-o-methylgalactose (R_{Gal} 4.8),

2, 6-di-o-methylgalactose (R_{Gal} 6.0) and 2, 3-di-o-methylgalactose (R_{Gal} 5.6). The presence of 4, 6-di-o-methylgalactose (R_{Gal} 4.8) (revealed with spray iii)was suspected. The mixture of di-o-methylgalactoses (9 mg) was converted into the corresponding alditol acetates and examined by g.l.c. at 160° with reference to the alditol acetates obtained from authentic di-o-methylgalactoses. The chromatogram revealed the presence of four di-o-methylgalactitol acetates with T_E 3.94 (identical to that from 2, 6-di-o-methylgalactose), T_E 4.27 (4, 6-di-o-methylgalactose), T_E 5.12 (2, 3-di-o-methylgalactose) and T_E 6.26 (2, 4-di-o-methylgalactose) in the approximate ratio 3:1:4:3. A further portion of fraction 2 was separated on paper (solvent d) to obtain chromatographically pure 2, 4-di-o-methylgalactose, $[\alpha]_D^{21}$ + 41° (c 0.44) (methanol). 2, 4-Di-o-methylgalactose (24 mg; 1 pt) was heated with aniline (3.5 pts) in ethanol (2 ml) containing a trace of acetic acid, for 4 h under reflux. The derived "anilide" which crystallised on cooling had, after recrystallisation from ethanol, m.p. 218-219°, $[\alpha]_D^{20}$ -193 → -187° (c 0.32; pyridine); lit.[90] 2, 4-di-o-methyl-D-galactose "anilide" m.p. 216°, $[\alpha]_D$ - 180° (c 1.5; pyridine).

Fraction 3. The syrup (338 mg) was a mixture of 2, 4, 6-tri-o-methylgalactose (major) and 2, 3, 6-tri-o-methylgalactose (paper chromatography). Chromatographically pure 2, 4, 6-tri-o-methylgalactose (265 mg) crystallised from an ether-light petroleum (1:1 v/v) solution of the syrup. Recrystallisation, from the same solvent, gave 2, 4, 6-tri-o-methylgalactose having m.p. 99-101°, and mixed m.p. 101-102° with an authentic sample,[91] $[\alpha]_D^{17}$ + 112° (4 min) → + 88° (c 0.75). The derived "anilide", after recrystallisation from ethanol, had m.p. and mixed m.p. 172-174° with authentic 2, 4, 6-tri-o-methyl-N-phenylgalactosylamine.[92] The molar

proportions of 2, 3, 6- and 2, 4, 6-tri-o-methylgalactose in the mother liquors (73mg) were determined by paper chromatography to be 1:2 by comparison with standard solutions.

Fraction 4. The syrup (609 mg) was shown by paper chromatography to contain 85% 2, 3, 6-tri-o-methylgalactose and 15% 2, 4, 6-tri-o-methylgalactose.

Fraction 5. The syrup (108 mg) which was chromatographically identical to 2, 3, 6 - tri-o-methylgalactose had $[\alpha]_D^{16}$ + 55° (4 min) ⟶ + 96° (c 0. 88). The syrup (36 mg) was oxidised[93] with bromine water (7 ml) for 4 days. After removal of the bromine by aeration, the solution was neutralized (Ag_2CO_3), filtered and the residual silver precipitated with hydrogen sulphide. The filtrate was evaporated to dryness in vacuo for 1h and extracted with dry ether. The colourless needles of 2, 3, 6-tri-o-methyl-D- ɣ -galactonolactone obtained on concentration of the extract had, after recrystallisation from dry ether, m. p. and mixed m. p. 98-99° with an authentic sample.[92]

Fraction 6. The syrup (20 mg) contained a mixture of 2, 3, 4, 6-tetra-o-methylgalactose (major) and 2, 3, 6-tri-o-methylgalactose (paper chromatography).

Fraction 7. The syrup (167 mg), $[\alpha]_D^{17}$ + 98° (final) (c 0. 53); $[\alpha]_D^{17}$ + 56° (final) (c 0. 67; ethanol) was chromatographically pure 2, 3, 4, 6-tetra-o-methylgalactose. The derived "anilide" had m. p. 192-194° which was not depressed when mixed with authentic 2, 3, 4, 6-tetra-o-methyl-N-phenyl-D-galactosylamine.[79]

2. 11 Periodate oxidation of polysaccharide and desulphated polysaccharide

To polysaccharide (32. 27 mg) and desulphated polysaccharide (14. 02 mg;

SO_4^{2-}, 0.9%) dissolved in water (5.00 ml) was added sodium metaperiodate (0.0294M; 5.00 ml). The solutions were set aside in the dark at room temperature and the consumption of periodate followed spectrophotometrically.[94] (Tables 1 and 2).

After oxidation had ceased the excess periodate was destroyed with ethylene glycol (0.5 ml). A complete hydrolysis of the dialysed material revealed the presence of galactose, 2-o-methylgalactose and 6-o-methylgalactose.

2.12 Methylation of the polysaccharide

Polysaccharide (10g; in the minimum quantity of water) under nitrogen, was stirred vigorously with sodium hydroxide solution (40% w/w; 200ml) and dimethyl sulphate (100 ml), the latter being added over 1 h. After 4 further additions of the above reagents, at two hourly intervals, the mixture was stirred overnight, dialysed against running water for 7 days and concentrated to a syrup. This procedure was carried out a further two times, after which the polymer (12.56g) was isolated by freeze-drying. (Found: OMe, 12.5%).

To a portion of the above partially methylated polymer (2.5g) in dimethyl sulphoxide (100ml) was added powdered sodium hydroxide (20g) and dimethyl sulphate (5ml) with vigorous stirring.[89] After 1 h a further aliquot of dimethyl sulphate (5ml) was added, and the mixture stirred vigorously overnight. The following day a second addition of reagents was made and the polymer (2.17g) isolated by freeze-drying after dialysis. (Found: OMe, 15.4%). Chromatographic investigation of an acid hydrolysate revealed traces of galactose, mono-o-methylgalactoses, plus several higher methylated products. Repetition of the above experiment

led to a negligible increase in methoxyl content. (Found: OMe, 15.5%) (Calculated for methylated polymer with 30% $SO_4^{=}$: OMe, 25.8%). The infrared spectrum ($CHCl_3$ solution) of this sample had a strong absorption peak in the O-H stretching region.

The partially methylated polymer was not soluble in Purdie's reagents[4] and it was not possible to further increase the methoxyl content.

2.13 Acetolysis of partially desulphated polysaccharide

Polysaccharide (39.6g; SO_4^{2-}, 30.3%) (dried in *vacuo* over P_2O_5 for 48h) was shaken with 0.15 M methanolic hydrogen chloride (21) for 60h at room temperature. The insoluble material was removed by centrifugation, dissolved in water and dialysed against distilled water for 7 days. The partially desulphated material (28.0g; SO_4^{2-}, 11.5%) was isolated by freeze-drying.

Dry partially desulphated polysaccharide (28.0g) was added to a mixture of acetic anhydride (155 ml), acetic acid (112 ml) and sulphuric acid (15.5 ml) with continual stirring, over a period of 0.5 h.[95] The resultant solution was shaken for 96 h at room temperature, clarified by centrifugation, mixed with ice-cold water (1.6 l) and neutralised ($NaHCO_3$) to congo red. The oligosaccharide acetates which precipitated were removed by filtration, and the solution extracted with chloroform (3 x 500 ml). The combined chloroform extracts and precipitate dissolved in chloroform, were dried (sodium sulphate), filtered, and evaporated to a syrup. Addition of water and freeze-drying gave a mixture of oligosaccharide acetates (31.2g).

Deacetylation[96] was carried out by vigorously stirring the mixture of oligosaccharide acetates (31.2g) with sodium methoxide (0.2M; 372ml)

for 1h at 25-30°. The suspension was made slightly acid with acetic acid, and water was added to dissolve the precipitated oligosaccharides. The solution was deionized by passage first through an Amberlite IR-120 (H^+) column (3 x 36 cm) and then through a column of Amberlite IRA 400 (CH_3COO^-) (3.7 x 30 cm), concentrated and the mixture of oligosaccharides isolated by freeze-drying (19.75g). Paper chromatography (solvent a) revealed numerous products with R_{Gal} <1, in addition to substances with the mobilities of galactose, and mono-methylgalactoses. The infrared spectrum (KBr disc) showed no ester carbonyl peak.

A portion (10.6g) of the oligosaccharide mixture was applied to a charcoal-Celite column (5.4 x 61 cm; 1:1 w/w), which was eluted with water (8l) and then with a linear gradient of aqueous ethanol (0-35%) over 58l. Fractions were collected, and on the basis of paper chromatography (solvents a and d) recombined into the following 13 fractions. The column was finally washed with 10% methyl ethyl ketone (5l).

Fraction 1. The partially crystalline syrup (2.46 g), eluted with water (8l) and aqueous ethanol (0-6.2%; 10.5l) was shown by paper chromatography to be a mixture of galactose, xylose (trace), 2-, 4-, and 6-o-methylgalactose.

Fraction 2. The chromatographically and electrophoretically pure syrup (239 mg) R_{Gal} 0.23 (solvent d), 0.36 (solvent a); M_G 0.66 (solvent f); D.P. 1.9, eluted with 6.2-6.9% aqueous ethanol (1.2l) had $[\alpha]_D^{20}$ + 150° (c 0.80). It gave only galactose on hydrolysis. The derived osazone,[97] after recrystallisation from water had m.p. 233-234° (lit:[95] 234-236° for 3-o- α -D-galactopyranosyl-D-galactosazone). The disaccharide (8 mg) in dimethyl sulphoxide (0.6 ml) and N,N- dimethylformamide (0.6 ml), was stirred with barium oxide (0.2g) and barium hydroxide octahydrate (0.1g)[98] under nitrogen with cooling. Dimethyl sulphate (0.3 ml) was added slowly and the mixture stirred for 24h at room temperature. Excess dimethyl

sulphate was destroyed with ammonia (0.3 ml), and the gel extracted with chloroform (5 x 3 ml). The chloroform extracts were washed with water (10 ml portions) until neutral and dried (magnesium sulphate). Methylation was found to be complete (t.l.c. spray iv). Examination by paper chromatography (solvent d, spray i) of an acid hydrolysate (0.5M H_2SO_4, 2h, 100°) of the methylated oligosaccharide revealed 2,3,4,6-tetra-o-methylgalactose and 2,4,6-tri-o-methylgalactose as the only reducing products. The methylated product was refluxed with 3% methanolic hydrogen chloride for 4h and the derived methyl glycosides examined by g.l.c. Peaks corresponding to 2,3,4,6-tetra-o-methylgalactose (T 1.79 column 1; 1.52, 1.58 column 2) and 2,4,6-tri-o-methylgalactose (T 4.26, 4.65 column 1; 2.97, 3.35 column 2) were observed. A further peak (T 4.41 column1; 3.24 column 2) was also present. This latter peak is considered to be a degradation product, arising as a result of the alkaline methylation conditions, since if the methylated methanolysed product is treated with Purdie's reagents[4] and then examined by g.l.c. (column 1) a peak with T 1.48 is obtained in addition to that of 2,3,4,6-tetra-o-methylgalactose (T 1.79). The oligosaccharide was methylated by a variety of methods viz., by the procedure of Haq and Percival,[5] completed by treatment with Purdie's reagents;[4] formation of the methyl glycosides by treatment with methanolic hydrogen chloride before methylation; and methylation as per Kuhn and Trischmann[98] but without N,N-dimethylformamide. In all cases the peak at T 4.41 occurred, to a varying extent. The disaccharide is assigned the structure 3-o- α -D-galactopyranosyl-D-galactose (1). The α-D configuration is assumed from the specific rotation of the compound.

Fraction 3. The syrup (421 mg) eluted with 6.9-9.2% aqueous ethanol (3.61) was shown by electrophoresis (solvent f) to be a mixture of 3-o- α -D-galactopyranosyl-D-galactose and a sugar electrophoretically equivalent to 4-o-β- D-galactopyranosyl-D-galactose (2). The latter which crystallised from the mixture when triturated with aqueous methanol had R_{Gal} 0.39 (solvent a), 0.26 (solvent d), M_G 0.53 (solvent f), $[\alpha]_D^{20}$ + 79° (3 min) → + 66° (c 0.53), m.p. 211-212° which was not depressed on admixture with 4-o- β -D-galactopyranosyl-D-galactose. The infrared spectrum (KBr disc) was identical to that obtained from an authentic sample of 4-o- β -D-galactopyranosyl-D-galactose.(84)

Fraction 4. The syrup (186 mg) eluted with 9.2-10.2% aqueous ethanol (2.01) contained two major components in addition to 4-o- β -D-galactopyranosyl-D-galactose. Separation of the syrup on Whatman No. 1 paper (solvent a, 3 days) and extraction of the appropriate portions of the papers with 50% aqueous methanol afforded each chromatographically pure.

Oligosaccharide 3. The syrup (56mg) R_{Gal} 0.62 (solvent a), 0.36 (solvent d), D.P. 2.0, $[\alpha]_D^{20}$ + 175° (c 0.67) gave galactose and 2-o-methylgalactose on partial acid hydrolysis (0.5M H_2SO_4, 15 min, 100°), and was revealed with spray (iii). It was chromatographically identical (solvents a-d) to the 3-o-(2-o-methylgalactopyranosyl)-galactose obtained on partial hydrolysis of the polysaccharide. An α-configuration is assumed from the optical rotation of the compound and the oligosaccharide is thus 3-o- α-(2-o-methyl-D-galactopyranosyl)-D-galactose.

Oligosaccharide 4. The syrup (12 mg) which had R_{Gal} 0.80 (solvent a), 0.44 (solvent d), D.P. 2.2, $[\alpha]_D^{20}$ + 7° (c 0.58) failed to react with spray (iii). Partial acid hydrolysis revealed (paper chromatography) the presence of galactose and 2-o-methylgalactose in addition to the original material, and, on total hydrolysis, only galactose and 2-o-methylgalactose, in approximately equal amounts, were obtained. Disaccharide (1 mg) in water

(1 ml) was reduced with sodium borohydride (5 mg) for 16 h. The solution was acidified with Amberlite IR-120 (H^+) resin, and the boric acid removed by distillation with methanol. Partial hydrolysis of the non-reducing syrup followed by paper chromatography revealed galactose as the only reducing sugar (spray i). A portion (2 mg) was methylated with dimethyl sulphate and barium oxide/hydroxide.[98] Hydrolysis with 0.5M sulphuric acid for 2 h followed by paper chromatography (solvent d) showed 2, 3, 4, 6-tetra-o-methylgalactose and 2, 3, 6-tri-o-methylgalactose only. Methanolysis followed by neutralisation with silver carbonate and analysis by g.l.c. showed peaks corresponding to 2, 3, 4, 6-tetra-o-methylgalactose (T 1.79 column 1; 1.53, 1.60 column 2) and 2, 3, 6-tri-o-methylgalactose (T3.32, 4.40, 4.72 column 1; 2.40, 2.95, 3.04, 3.33 column 2). The above results indicate that the oligosaccharide is a 4-o-galactopyranosyl-2-o-methylgalactose. The disaccharide is chromatographically distinguishable (solvents a and d) from 4-o- β -D- galactopyranosyl-2-o-methyl-D-galactose. It is tentatively suggested that one of the monomers occurs as the L-isomer.

Fraction 5. The syrup (146 mg) eluted with aqueous ethanol (10.2-10.8%; 1.11) was found by paper chromatography to be a mixture of Fractions 4 and 6.

Fraction 6. The syrup (401 mg) eluted with 10.8-12.2% aqueous ethanol (2.31) was shown (paper chromatography) to contain 2 major oligosaccharides. Separation of a portion (202 mg) of the syrup on Whatman No. 1 paper (solvent d; 4 days) followed by extraction of the relevant portions of the paper with methanol- water (1:1) yielded the following two products.

Oligosaccharide 5. The chromatographically pure syrup (80 mg) R_{Gal} 0.75 (solvent a), 0.52 (solvent d), D.P. 2.1 had $[\alpha]_D^{20}$ + 188° (c 0.50). Galactose and 6-o-methylgalactose were produced on partial acid hydrolysis of a sample, while reduction followed by hydrolysis yielded galactose as the only reducing sugar. A portion (4.4mg) was methylated using the procedure of Kuhn and Trischmann.[98] Paper chromatographic examination of a hydrolysate of the methylated product revealed 2,3,4,6-tetra-o-methylgalactose and 2,4,6-tri-o-methylgalactose. This was confirmed by t.l.c. (spray iv). The methanolysed product gave peaks on g.l.c. equivalent to 2,3,4,6-tetra-o-methylgalactose (T 1.78 column 1; 1.53, 1.59 column 2) and 2,4,6-tri-o-methylgalactose (T 4.18, 4.66 column 1; 2.98, 3.36 column 2). The peak at T 4.41 (column 1), obtained on methylation of 3-o- α-D-galactopyranosyl-D-galactose, was also present. The oligosaccharide is assigned the structure 3-o- α-D-galactopyranosyl-6-o-methyl-D-galactose, the α-configuration being assumed from the optical rotation.

Oligosaccharide 6. The crystalline disaccharide (48 mg) R_{Gal} 0.96 (solvent a), 0.76 (solvent d), $[\alpha]_D^{20}$ + 85° (4 min) → + 70° (c 0.47) had m.p. 217-218° which was undepressed on admixture with 4-o-β-D-galactopyranosyl-2-o-methyl-D-galactose. The two compounds had identical infrared spectra (KBr disc).

Fraction 7. The fraction (412 mg) eluted with aqueous ethanol (12.2-14.2%; 3.21) was shown by paper chromatography to contain the oligosaccharides of fraction 8 and a substance R_{Gal} 0.96 (solvent a), 0.68 (solvent d), chromatographically identical to 4-o-β-(6-o-methyl-D-galactopyranosyl)-D-galactose (7).

Fraction 8. The syrup (361 mg) eluted with 14.2-15.7% aqueous ethanol (2.91) crystallised from methanol on standing. The oligosaccharide (8)

(143 mg) R_{Gal} 0.09 (solvent a), 0.06 (solvent d), D.P. 3.0 had $[\alpha]_D^{20}$ +130° (c 0.60) and m.p. 233-235° after recrystallisation from methanol. On partial acid hydrolysis (0.5M H_2SO_4, 15 min, 100°) the oligosaccharide gave products electrophoretically identical to oligosaccharides 1 and 2 in addition to galactose, while total hydrolysis yielded galactose only. Partial hydrolysis of a sample (2 mg) after borohydride reduction gave oligosaccharide 2 as the only reducing disaccharide. Methylation[98] and methanolysis of a further reduced sample (5mg) showed on g.l.c. peaks corresponding to 2,3,4,6-tetra-o-methylgalactose, 2,3,6-tri-o-methylgalactose and a penta-o-methylgalactitol (T 0.54, column 1). The oligosaccharide (5 mg) was methylated with barium oxide/hydroxide[98] under nitrogen, hydrolysed and examined by paper chromatography (solvent d), and t.l.c. In both cases spots having the mobilities of 2,3,4,6-tetra-o-methylgalactose, 2,3,6-tri-o-methylgalactose and 2,4,6-tri-o-methylgalactose were obtained. A methanolysed sample gave on g.l.c. analysis peaks corresponding to 2,3,4,6-tetra-o-methylgalactose (T 1.78 column 1; 1.53, 1.59 column 2) and the mixture of tri-o-methylgalactoses (T 3.32, 4.41, 4.70 column 1; 2.39, 3.01, 3.32 column 2). The oligosaccharide is thus assigned the structure o- β -D-galactopyranosyl- (1→4)-o- α -D-galactopyranosyl-(1→3)-D-galactose.

The supernatant solution was concentrated to a syrup (132 mg) and separated in solvent a (Whatman No. 1 paper) for 10 days. Extraction of the relevant portions of paper with 50% aqueous methanol yielded pure oligosaccharide (9) (14 mg) R_{Gal} 0.14 (solvent a), 0.06 (solvent d), $[\alpha]_D^{20}$ + 126° (3 min) → + 146° (c 0.38) which readily crystallised from aqueous ethanol, and after recrystallisation from the same solvent had m.p. 232-233°. On partial hydrolysis galactose, oligosaccharides 1

and 2 were obtained (electrophoresis, solvent f) while partial hydrolysis of a reduced sample gave oligosaccharide 1 as the only reducing disaccharide. Thus the oligosaccharide is o- α -D-galactopyranosyl-(1→3) -o- β -D-galactopyranosyl - (1→4)- D-galactose.

Fraction 9. The syrup (277 mg) eluted with 15.7-17.3% aqueous ethanol (2.81) contained 4 major oligosaccharides. Separation in solvent a (7 days) yielded the following chromatographically pure products.

Oligosaccharide 10. The syrup (6 mg) had R_{Gal} 0.05 (solvent a), 0.01 (solvent d), D.P. 4.0 and $[\alpha]_D^{20}$ + 115° (c 0.71). Paper chromatography of a total hydrolysate gave a product with the mobility of galactose. On partial hydrolysis, products corresponding to oligosaccharides 1, 2, 8 and 9 were obtained in addition to the original sugar (solvents a and f). Reduction of a portion followed by partial hydrolysis and paper chromatography showed the presence of oligosaccharides 1, 2 and 8. The above evidence indicates that oligosaccharide 10 is o- β -D-galactopyranosyl-(1→4)-o- α -D-galactopyranosyl-(1→3)-o- β -D-galactopyranosyl-(1→4)-D-galactose.

Oligosaccharide 11. The syrup (6.5 mg) R_{Gal} 0.22 (solvent a), 0.05 (solvent d), $[\alpha]_D^{20}$ + 100° (c 0.50) which was revealed with spray (iii) gave on partial hydrolysis oligosaccharides 3 and 6 in addition to galactose and 2-o-methylgalactose; and on reduction followed by partial hydrolysis galactose, 2-o-methylgalactose and oligosaccharide 6. Thus the oligosaccharide is assigned the structure o- β -D-galactopyranosyl-(1→4)-o- α -(2-o-methyl-D-galactopyranosyl)-(1→3)-D-galactose.

Oligosaccharide 12. The sugar (14mg) which crystallised from methanol had R_{Gal} 0.25 (solvent a), 0.11 (solvent d), $[\alpha]_D^{20}$ + 142° (c 0.55), D.P. 3.0, m.p. 263-264° and was revealed with spray (iii). Partial acid hydrolysis produced oligosaccharides 2 and 3, galactose and

2-o-methylgalactose, and hydrolysis of the derived oligosaccharide alcohol gave galactose, 2-o-methylgalactose and oligosaccharide 3 as the reducing products. A portion (3 mg) was methylated by the method of Kuhn and Trischmann.[98] Paper chromatographic (solvent d) and t.l.c. examination of the acid hydrolysate revealed spots with the mobilities of 2,3,4,6-tetra-o-methylgalactose, 2,3,6-tri-o-methylgalactose and 2,4,6-tri-o-methylgalactose. This trisaccharide is thus o- α -(2-o-methyl-D-galactopyranosyl)-(1→3)-o- β -D-galactopyranosyl-(1→4)-D-galactose.

Oligosaccharide 13. The syrup (56 mg) which was not revealed with spray (iii) had R_{Gal} 0.34 (solvent a), 0.16 (solvent d), $[\alpha]_D^{20}$ + 153° (c 0.45), D.P. 2.9. On partial acid hydrolysis it gave oligosaccharides 1 and 6, galactose and 2-o-methylgalactose and on total hydrolysis galactose and 2-o-methylgalactose in the approximate ratio 2:1. Treatment of a portion (2 mg) with sodium borohydride followed by partial acid hydrolysis gave oligosaccharide 1 and galactose as the only reducing products. Methylation followed by hydrolysis and paper chromatography (solvent d) and by methanolysis and g.l.c. showed 2,3,4,6-tetra-o-methylgalactose, 2,3,6-tri-o-methylgalactose and 2,4,6-tri-o-methylgalactose or their glycosides respectively. Thus the oligosaccharide is assigned the structure o- α -D-galactopyranosyl-(1→3)-o- β -D-galactopyranosyl-(1→4)- 2 -o-methyl-D-galactose.

Fraction 10. The syrup (305 mg) eluted with aqueous ethanol (17.3-18.7%; 2.11) was shown by chromatography to be a mixture of several oligosaccharides. The two major components were obtained by paper chromatographic separation of a portion (94 mg) in solvent d (3 days).

Oligosaccharide 14. The syrup (4 mg) which had R_{Gal} 1.97 (solvent a),

1.70 (solvent d) gave 2-o-methylgalactose and 6-o-methylgalactose on partial acid hydrolysis, and only 6-o-methylgalactose on hydrolysis following reduction. This oligosaccharide is chromatographically identical to 4-o- β -(6-o-methyl-D-galactopyranosyl)-2-o-methyl-D-galactose obtained from the partial hydrolysis studies.

The syrup (47 mg) R_{Gal} 0.34 (solvent a), 0.16 (solvent d) was chromatographically and electrophoretically homogeneous in all solvents. Acid hydrolysis of the fraction produced galactose, 2-o-methylgalactose and 6-o-methylgalactose, and on partial hydrolysis the oligosaccharides 1, 2, 5 and 6 in addition to the above monomers. Reduction followed by partial acid hydrolysis and examination by electrophoresis (solvent f) gave oligosaccharides 1 and 2 and galactose (spray ii). The above results can be explained by assuming the fraction to be a mixture of oligosaccharide 13, o- α -D-galactopyranosyl-(1 → 3)-o- β -D-galactopyranosyl-(1 → 4)-2-o-methyl-D-galactose, and o- β -D-galactopyranosyl-(1→ 4)-o- α -D-galactopyranosyl-(1 → 3)-6-o-methyl-D-galactose (15).

Fraction 11. The syrup (357 mg) eluted with aqueous ethanol (18.7-20.4%; 2.81) contained several slow moving products (paper chromatography) and was not further investigated.

Fraction 12. The syrup (292 mg) was eluted with 20.4-22.1% aqueous ethanol (2.91). The major component (26 mg) was obtained by fractionation of a portion (136 mg) on Whatman No. 1 paper (solvent a, 6 days) and had R_{Gal} 0.53 (solvent a), 0.28 (solvent d), D.P. 2.8, $[\alpha]_D^{20}$ + 126° (c 0.46); it was not revealed with spray (iii). Partial acid hydrolysis of a portion yielded oligosaccharides 3 and 6, galactose and 2-o-methylgalactose, while partial hydrolysis after reduction gave oligosaccharide 3, galactose and 2-o-methylgalactose. Methylation followed by hydrolysis

yielded 2, 3, 4, 6-tetra-o-methylgalactose, 2, 3, 6-tri-o-methylgalactose and 2, 4, 6-tri-o-methylgalactose (paper chromatography and t. l. c.). These results indicate that the oligosaccharide is o- α -(2-o-methyl-D-galactopyranosyl)-(1→3)-o - β -D-galactopyranosyl-(1→4)-2-o-methyl-D-galactose. (16)

Fraction 13. The fraction (1. 44g) eluted with aqueous ethanol (22-35%; 17. 71) was shown by paper chromatography to contain several slow moving saccharides ($R_{Gal} < 0.1$, solvents a and d). It was not further investigated.

Fraction 14 (1. 56g) was eluted with aqueous methyl ethyl ketone and shown to contain polymeric material (paper chromatography). The fraction was not further investigated.

3. DISCUSSION

Pachymenia carnosa (J. Ag.) J. Ag., a red seaweed of the Grateloupiaceae family, is a cold water alga which grows in the lowest part of the tidal range. The thallus consists of irregularly lobed, flattened straps which have a tough leathery texture and are deep red in colour. The weed used in the present investigation was collected near Cape Town, South Africa.

Extraction of P. carnosa with hot water followed by centrifugation and precipitation of the mucilage into ethanol afforded a highly sulphated, methylated polysaccharide, purification of which was effected by dissolution in water followed by centrifugation and subsequent precipitation into ethanol. The infrared spectrum of the polymer (Fig. 1a) showed an ester sulphate peak[99] at 1240 cm^{-1}, but there was no resolution into discrete peaks of the broad peak centered at 825cm^{-1}, thus giving no indication of the type of ester sulphate present.

The polysaccharide was totally hydrolysed with acid and the component sugars separated on a charcoal-Celite column using water and aqueous ethanol. D-Galactose, 2-o-methyl-D-galactose and 6-o-methyl-D-galactose were obtained, all in crystalline form, and characterised by optical rotation, melting point and mixed melting point with authentic samples. The 4-o-methylgalactose obtained was characterised as the 4-o-methyl-N-phenylgalactosylamine. The low optical rotation of this sugar indicates that it occurs as a mixture of the D- and L- isomers. It is of interest to note that both 4-o-methyl-D-galactose[100] and 4-o-methyl-L-galactose[92] have been found as constituents of sulphated polysaccharides extracted from Grateloupiaceae species but this is the first instance where both isomers have been obtained

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

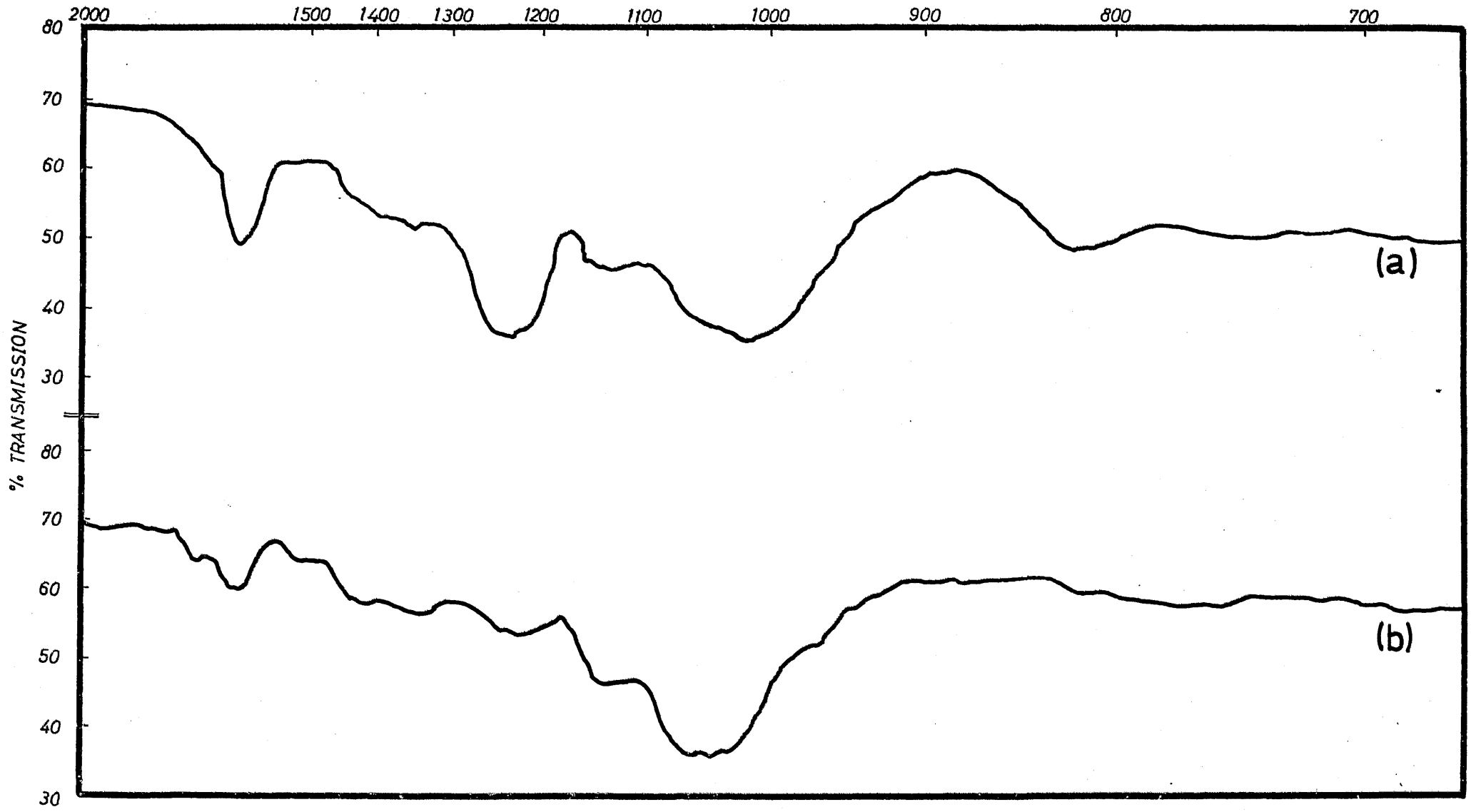


Fig.1

in one polymer. The isolation of 4-o-methyl-DL-galactose implies that metabolic pathways must be present in Grateloupiaceae species for the synthesis of both isomers, and helps clarify a possible source of controversy.

Quantitative estimation of the component sugars was carried out using the derived alditol acetates.[82] This derivative was chosen because of its ease of preparation and since only one peak is given on g. l. c. by each component sugar, which simplifies quantitative measurements. Erythritol was used as the internal standard as it is not a constituent part of the polymer, and the retention time of its acetate was very different from that of the alditol acetates derived from the component sugars. All alditol acetates do not have the same molar response on g. l. c. so this factor had to be determined with the aid of standard solutions of the component monomers. It was found that the molar responses of the three mono-o-methyl-galactoses were similar, but differed from those of galactose and xylose. The reason the minute amount of xylose could be determined is because xylitol pentaacetate has a short retention time, which increases sensitivity.

The method of determining sulphate content with 4'-chlorobiphenyl-4 -ylamine[75, 76] involves converting the sulphate in the polymer to ionic sulphate by combustion, which is then complexed with the reagent. The amount of sulphate present is determined from the decrease in absorbance of the reagent with reference to a standard calibration curve. The amount of material utilized is inversely proportional to its sulphate content.

The molar ratio of the components, (galactose: 2-o-methylgalactose: 6-o-methylgalactose: 4-o-methylgalactose: xylose: $NaSO_3^-$) was determined to be 6. 15: 1. 0: 0. 52: 0. 21: 0. 03: 6. 59. It is possible to

calculate the methoxyl content of the polysaccharide from these values. This value (2.5%) is slightly higher than that obtained experimentally using the Zeisel method[101] (2.2%). The discrepancy probably arises from an over-estimate of the 4-o-methylgalactose content. The peak attributed to the alditol acetate of 4-o-methylgalactose was incompletely resolved from the peak from 2-o-methylgalactose, and this complicated estimation of the peak area. If the polymer did contain as much as 2% 4-o-methylgalactose one would have expected this to be reflected in the hydrolysis and acetolysis products. The small amount of xylose present is considered to be a contaminant.

Alkali does not cause S-O cleavage but can eliminate sulphate if (a) a hexose is sulphated on position-6 and has a free hydroxyl group on position-3 (or vice versa) or (b) there is a free hydroxyl group adjacent and trans to the sulphate group.[102] In the first case the 3,6-anhydrohexose will be formed, and in the second an epoxide ring is produced. A base such as sodium methoxide can break the epoxide ring and an epimeric pair of mono-o-methyl sugars will be formed, with the methoxyl groups trans to the former epoxide ring. The polysaccharide was treated with base in the presence of sodium borohydride,[103] the latter being added to reduce end-group degradation. Only a negligible quantity (1%) of 3,6-anhydrogalactose was obtained on treatment with sodium hydroxide and no new monomethyl- or dimethyl- sugar was obtained after reaction with sodium methoxide. The large amount of sulphate (30.3%) in the polymer suggests that the reason neither of the above conditions for alkali lability is satisfied is because there are no suitably situated free hydroxyl groups- these being blocked by methyl ethers, glycosidic linkages and/or sulphate (i.e. multisulphated units).

It would be of interest to see whether a larger amount of 3, 6-anhydro-galactose and/or epoxide rings could be formed if the polymer were first partially desulphated. Elimination of a portion of the sulphate groups by S-O cleavage could give rise to the necessary conditions for anhydride formation.

Partial acid hydrolysis of the polysaccharide, followed by separation of the neutral products on a charcoal-Celite column yielded, in addition to the monosaccharides above, several disaccharides and an, as yet, unidentified monosaccharide. The only definite results obtained for this monomer (R_{Gal} 3.70, solvent a) are that it is unchanged on hydrolysis, is a reducing sugar, and is substituted in position-2. Two products were obtained on treating the sugar with hydrobromic acid; one is chromatographically identical with galactose and the other does not correspond to a monomethylgalactose. On exhaustive methylation a product with the mobility of 2, 4, 6-tri-o-methylgalactose was obtained.

Only two disaccharides obtained from the partial hydrolysis were crystalline, namely 4-o- β -D-galactopyranosyl-D-galactose and 4-o- β -D-galactopyranosyl-2-o-methyl-D-galactose. Other disaccharides (all chromatographically pure, but non-crystalline) characterised were 3-o-(2-o-methyl-D-galactopyranosyl)-D-galactose, 4-o- β -(6-o-methyl-D-galactopyranosyl)-D-galactose, 4-o- β -(6-o-methyl-D-galactopyranosyl)-2-o-methyl-D-galactose and a 2-o-methylgalactopyranosyl-6-o-methyl-galactose. These oligosaccharides were characterised using the following general procedure. The component sugars were determined by a total hydrolysis of the oligosaccharide and investigation of the hydrolysate by paper chromatography. The order of the component sugars

was determined by reduction followed by hydrolysis. The reducing end-group is converted into the glycitol and thus only the non-reducing end-group sugar is revealed with a spray which is dependent upon reducing properties. The position of the glycosidic linkage was determined by methylation. In all cases 2, 3, 4, 6- tetra-o-methylgalactose was obtained from the non-reducing end-group, while the reducing end-group yielded either 2, 3, 6-tri-o-methylgalactose, indicating a (1→4) glycosidic linkage or 2, 4, 6-tri-o-methylgalactose, from a (1→3) linkage. There are two methods available for determining the methylation products, paper chromatography after hydrolysis or g. l. c. examination of a methanolysate. The latter is preferable as detection does not depend on a chemical property and all products will be revealed. This will indicate whether degradation has occurred during the methylation procedure. Methylation masks the presence of any monomethyl sugar in a disaccharide but this is of little consequence as its position would already have been evident. The anomeric configuration was determined by Hudson's rules of isorotation.[63] A superior method would be to determine the chemical shift of the glycosidic anomeric proton; a strong singlet characteristic of either an α or β proton is obtained in addition to the two signals from the reducing anomeric proton.[64]

The polysaccharide was partially hydrolysed with acid and the sulphated sugars separated from the neutral material with the aid of ion-exchange chromatography. It was found that the charged sugars do not run well on paper chromatograms, streaking badly, and are not cleanly eluted from charcoal columns. Repeated separations of the complex mixture of acidic products yielded only one chromatographically pure compound, identified as D-galactose-6-sulphate.

This compound was hydrolysed to galactose, gave 2, 3, 4- tri-o-methylgalactose on methylation, had a DP of 1.0 and was chromatographically and electrophoretically identical to L-galactose-6-sulphate. The D-isomer is indicated by the optical rotation which was calculated from the galactose content, determined by the method of Dubois et al.[86] The only anomolous evidence was the large amount of sulphate present, which is attributed to contaminating ionic sulphate. The initial galactose: sulphate molar ratio of 1:6.2 was decreased to 1:2.8 by shaking an aqueous solution of this sugar, in the free acid form, with a 5% chloroform solution of tri-n-octylamine.[87] The tertiary amine acts as an ion-exchanger, removing ionic sulphate from the aqueous solution by salt formation. The acidic sugar remains in the aqueous solution due to the presence of the hydrophilic sugar residue. Unfortunately insufficient material remained for the procedure to be repeated until a constant molar ratio of galactose:sulphate was obtained. That such a large proportion of the sulphate could be removed by one treatment with the reagent eliminates the possibility that it has structural significance. As the sulphate in the polysaccharide is stable to alkali the D-galactose-6-sulphate can exist in the polymer as a (1 → 3) linked unit or, if it is (1 → 4) linked, position-3 must also be blocked, either by a glycosidic linkage, implying a branch point, or by a second sulphate group. If the blocking group is sulphate it is necessary to postulate a tri-substituted (1 → 4) linked unit for all the conditions of alkali stability to be fulfilled.

The modes of glycosidic linkage in a polymer are generally determined by investigating the hydrolysis products of a fully methylated sample - the positions of free hydroxyl groups in the derived monomers indicating the type of glycosidic linkage. For sulphated polysaccharides the

situation is complicated. Methylation and hydrolysis of the sulphated polysaccharide will give the positions of substitution, both glycosidic and sulphate, and methylation of a sample free of sulphate is necessary to determine the types of glycosidic linkage present. The standard reagent for the removal of sulphate groups is anhydrous methanolic hydrogen chloride.[88] The sulphate groups are removed by S-O cleavage but, unfortunately, some glycosidic bonds are also broken. This must be borne in mind when results are transposed to the native polymer.

The polysaccharide was desulphated with relative ease, the most efficient conditions found were shaking the sample with a 0.15M solution of methanolic hydrogen chloride for 48 h at room temperature. This resulted in the sulphate content being decreased to 1.7% (78% yield). The infrared spectrum (Fig. 1b) of this sample showed a much reduced peak at 1240cm^{-1} (due to ester sulphate), while the peak at 820-850 cm^{-1} had completely disappeared. A second treatment with 0.15M methanolic hydrogen chloride yielded a sample with a sulphate content of 0.8%. Desulphation of a larger amount of material for methylation and other studies was carried out under the same conditions, three treatments with the reagent being necessary to obtain a "sulphate free" sample (Found: SO_4^{2-}, 0.9%; 53% yield). The degradation resulting from treatment with methanolic hydrogen chloride appears to be non-specific as hydrolysis of the non-reducing methanol-soluble material gave (on paper chromatography the same components, in essentially the same ratio, as a hydrolysate of the native polysaccharide.

As the desulphated polymer was soluble in dimethyl sulphoxide, methylation was carried out using the method of Srivastava et al.[89] After 3 such treatments the polysaccharide was extracted with chloroform. The first chloroform extract which comprised 63% of the methylated

product showed a very small peak in the 2800-3300 cm^{-1} region of the infrared spectrum. It was found to have a methoxyl content of 43.4%, which could only be increased to 43.8% on treatment with Purdie's reagents.[4] (Theoretical for a methylated hexan containing 1% SO_4^{2-}; OMe, 44.2%).

Highly methylated polysaccharides cannot be hydrolysed in the normal manner due to their insolubility in dilute mineral acid. One of the methods available for hydrolysis is a partial formylysis, followed by total hydrolysis with dilute mineral acid. This approach causes minimal degradation and demethylation, and was used to hydrolyse the methylated, desulphated polymer. The hydrolysate was separated into its component sugars on a charcoal-Celite column using aqueous methyl ethyl ketone as eluting agent. The main products were found to be 2,3,6-tri-o-methyl-D-galactose and 2,4,6-tri-o-methyl-D-galactose (ca 80% of the methylated units isolated) both characterised as crystalline derivatives by comparison with authentic samples. The presence of these two products, in almost equal amounts, complements the results obtained from the partial hydrolysis and acetolysis studies. Only oligosaccharides containing (1→3) and (1→4) glycosidic linkages have been obtained and in the tri- and higher-saccharides an alternating sequence of (1→4) and (1→3) linked units is indicated (see later). From the relatively large amount of 2,3,4,6-tetra-o-methylgalactose obtained, it appears that degradation occurred during the desulphation procedure, resulting in the formation of shorter polysaccharide chains. If extensive branching was present it would be expected that the di-o-methylgalactose fraction would contain chiefly one component. This did not, in fact, occur; the presence of 2,6-, 4,6-, 2,3- and 2,4-di-o-methylgalactoses in the approximate ratio 3:1:4:3 was demonstrated by g.l.c. investigation

of their alditol acetates. These components, and the small amount of 2,3,4-tri-o-methylgalactose obtained, are considered to be undermethylation products, or to have arisen by demethylation during hydrolysis. No galactose or monomethylgalactose was obtained.

Samples of native and desulphated polymer were independently oxidised with sodium metaperiodate and the amount of periodate consumed followed spectrophotometrically.[94] (Tables 1 and 2).

TABLE 1: Moles Periodate Reduced per Anhydro Hexose Residue

Time (h)	4	12	24	48	72	96
P. carnosa ($SO_4^=$, 30.3%)	0.040	0.059	0.095	0.107	0.130	0.129
Desulphated polysaccharide ($SO_4^=$, 0.9%)	0.078	0.182	0.232	0.264	0.328	0.329

TABLE 2: Moles Periodate Reduced per Sulphate Free Anhydro Hexose Residue

Time (h)	4	12	24	48	72	96
P. carnosa	0.057	0.085	0.136	0.154	0.186	0.185
Desulphated polysaccharide	0.079	0.184	0.234	0.266	0.331	0.332

For direct comparison results from Table 2 are more significant since allowance has been made for the sulphate contents of the two polysaccharides.[84] Consumption of periodate ceased after 72 h when 0.185 and 0.331 moles of periodate had been consumed per sulphate free anhydrohexose unit in the polysaccharide and desulphated sample, respectively.

The low consumption of periodate by the native polymer can be accounted for by a) the presence of (1→3) glycosidic linkages and b) blocking of some of the other glycosidic linkages by methoxyl and/or sulphate groups and/or branches. From the partial hydrolysis and acetolysis studies, and because the major products obtained on methylating a desulphated sample were 2, 3, 6-tri-o-methylgalactose and 2, 4, 6-tri-o-methylgalactose, it appears that the only other major glycosidic linkage is (1→4). 2-o-Methylgalactose occurs in the polysaccharide linked through position-4, and would thus render those units immune to periodate attack. The higher consumption of periodate by the desulphated sample must be interpreted with caution because of the degradation which occurred during the desulphation process. However, it is probable that some of the additional uptake of periodate is due to an increase in the quantity of α-glycol groupings, since a small amount (1%) of 3, 6-anhydrogalactose is formed on treating the polysaccharide with alkali. If this alkali-labile sulphate is situated at position-3 of a (1→4) linked galactose unit, that unit would, on desulphation, be cleaved by periodate.

Acid hydrolysis of both periodate oxidised polysaccharides revealed the presence of galactose, 2-o-methylgalactose and 6-o-methylgalactose. That the 4-o-methylgalactose is cleaved by periodate indicates these units must either be linked through position-6, or occur as end-groups in the polysaccharide chain. No oligosaccharides containing 4-o-methylgalactose were obtained from the partial hydrolysis or acetolysis investigations so it is not possible to distinguish between these two possibilities. In the polysaccharide from Aeodes ulvoidea (Grateloupiaceae) the major monomethyl sugar is 4-o-methyl-L-galactose and it, too, is cleaved by periodate. Allsobrook et al[92] have suggested that the 4-o-methylgalactose probably occurs as a non-reducing end-group because a) very little

2, 3, 4-tri-o-methylgalactose was obtained from a methylated, desulphated polymer and b) the 2, 3, 4, 6-tetra-o-methylgalactose obtained was a racemate, and the only "L-galactose" in the polymer occurs as the 4-o-methyl ether. The immunity of the 2-o-methyl- and 6-o-methyl-galactose to periodate attack can be predicted by considering the results of the partial hydrolysis and acetolysis studies: the 2-o-methyl ether occurs linked glycosidically through positions 1 and 4 while the 6-o-methylgalactose is (1→3) linked. Some of the galactose units are resistant to periodate attack being (1→3) linked.

In order to obtain an indication of the position of the sulphate esters an attempt was made to methylate the polysaccharide with the sulphate groups intact. The highest methoxyl content which could be achieved was 15.5%, 60% of the theoretical value for a fully methylated sample. The resistance to methylation is attributed to the blocking action of the bulky sulphate group, and to its hydrophilic nature compared with the hydrophobic methoxyl group; thus the partially methylated sample was not soluble in either aqueous or organic solvents. In general, it has been found that complete methylation of all the free hydroxyl groups in a sulphated polymer can only be achieved if the sulphate content is fairly low. In view of this low methoxyl value no investigations were carried out on the methylated, sulphated sample, as any results obtained would not have been significant.

Different glycosidic linkages have different susceptibilities to attack by chemical reagents; it being known, for example, that (1→4) glycosidic linkages are much more stable to acid attack than (1→3) linkages. Acetolysis and partial hydrolysis complement each other in this respect with (1→3) linkages being more stable to acetolysis. Thus an acetolysis was carried out on the polysaccharide in order to

obtain further information about its fine structure. It was decided to use a partially desulphated sample for this investigation as this would minimise the effect of sulphate on the cleavage pattern and possibly enable one to obtain a truer picture of the repeating units present.

After acetolysis and deacetylation, acidic material was removed from the mixture by means of an ion-exchange resin. The neutral oligosaccharides were fractionated on a charcoal-Celite column and separation by paper chromatography of these fractions led to the isolation and identification of 16 oligosaccharides. The method used to elucidate the structures of these oligosaccharides was essentially that employed on the disaccharides obtained as products of the partial hydrolysis. In addition, to find the relative order of the monomers in a tri- or tetra- saccharide the oligosaccharide was hydrolysed for 15 min with dilute acid. The disaccharides obtained were characterised by their chromatographic and/or electrophoretic mobilities. A similar partial hydrolysis was carried out on the reduced oligosaccharide, in preference to a total hydrolysis. Identification of the methylation products of a tri- or tetra- saccharide by g. l. c. was very unsatisfactory as the peaks from 2, 3, 6- and 2, 4, 6- tri-o-methylgalactose overlap. However, these two tri-o-methylgalactoses are easily distinguishable on paper chromatograms. In these cases g. l. c. was used only to determine whether there were any other products present. The first peak of methyl 2, 3, 6- tri-o-methylgalactoside (T3. 3, column1) is the only discrete peak obtained from the tri-o-methylgalactose mixture. This peak could be used to calculate the proportions of the two tri-o-methyl sugars present if the anomeric and ring isomers from 2, 3, 6-tri-o-methylgalactoses are always produced in the same ratio. This is not the case, however, this ratio is dependent

on factors such as the method of methylation and time of methanolysis. To a certain extent the proportions of these isomers are characteristic of the parent sugar.

An additional peak was obtained on g. l. c. investigation of methylated (1→3) linked disaccharides. This peak (T4.41, column 1) appears to be a non-reducing product as no extra products were revealed by paper chromatography or t. l. c. (spray i) - both showing only products corresponding to 2, 3, 4, 6-tetra-o-methylgalactose and 2, 4, 6-tri-o-methylgalactose. Evidence that this was a degradation product was obtained by methylating a methanolysed sample of oligosaccharide 1. Two peaks were obtained on g. l. c.; a peak at T1.48 (column 1) and one equivalent to methyl 2, 3, 4, 6-tetra-o-methylgalactosides, while the peaks corresponding to 2, 4, 6- tri-o-methylgalactose disappeared. If the peak at T4.41 had been a galactose derivative only 2, 3, 4, 6- tetra-o-methylgalactose would have been present after the second methylation. The nature of the product (T4.41) is not known, but it probably arises from alkaline degradation of the reducing end-group as a result of the highly basic methylation conditions.

It is not obvious whether degradation occurs during methylation of (1→4) linked units, as T4.41 corresponds to one of the peaks given by 2, 3, 6-tri-o-methylgalactose. However, methylation of a methanolysate would clarify the position.

A list of the oligosaccharides which have been characterised from the sulphated polysaccharide of Pachymenia carnosa together with a résumé of the structural evidence is given in Table 3. Oligosaccharides (2), (6), (8), (9), (12) were obtained crystalline.

TABLE 3: Oligosaccharides of P. carnosa

Oligosaccharide		Total hydrolysis products	Partial hydrolysis products	Partial hydrolysis products (after reduction)	Hydrolysis or methanolysis products of methylated oligosaccharide	D. P.
$G_D\,\alpha\,(1\rightarrow3)G_D$	(1)	G			A, B	1.9
$G_D\,\beta\,(1\rightarrow4)G_D$	(2)	G			A, C 1.0:1.0	
$2_D\,\alpha\,(1\rightarrow3)G_D$	(3)	G, 2	G, 2	2	A, B	2.0
$G(1\rightarrow4)2$	(4)	G, 2	G, 2	G	A, C	2.2
$G_D\,\alpha\,(1\rightarrow3)6_D$	(5)		G, 6	G	A, B	2.1
$G_D\,\beta\,(1\rightarrow4)2_D$	(6)	G, 2	G, 2	G	A, C 1:1.1	
$6_D\,\beta\,(1\rightarrow4)G_D$	(7)	G, 6	G, 6	6	A, C 1:1.0	
$G_D\,\beta\,(1\rightarrow4)G_D\,\alpha\,(1\rightarrow3)G_D$	(8)	G	G, (1), (2)	G, (2)	A, B, C	3.0
$G_D\,\alpha\,(1\rightarrow3)G_D\,\beta\,(1\rightarrow4)G_D$	(9)		G, (1), (2)	G, (1)		
$G_D\,\beta\,(1\rightarrow4)G_D\,\alpha\,(1\rightarrow3)G_D\,\beta\,(1\rightarrow4)G_D$	(10)	G	G, (1), (2), (8), (9)	G, (1), (2), (8)		4.0
$G_D\,\beta\,(1\rightarrow4)2_D\,\alpha\,(1\rightarrow3)G_D$	(11)		G, 2, (3), (6)	G, 2, (6)		
$2_D\,\alpha\,(1\rightarrow3)G_D\,\beta\,(1\rightarrow4)G_D$	(12)		G, 2, (2), (3)	G, 2, (3)	A, B, C	3.0
$G_D\,\alpha\,(1\rightarrow3)G_D\,\beta\,(1\rightarrow4)2_D$	(13)	G, 2	G, 2, (1), (6)	G, (1)	A, B, C	2.9
$6_D\,\beta\,(1\rightarrow4)2_D$	(14)		2, 6	6	A, C 1:1.1	
$G_D\,\beta\,(1\rightarrow4)G_D\,\alpha\,(1\rightarrow3)6_D$	(15)	G, 6	G, 6, (5), (2)	G, (2)		
$2_D\,\alpha\,(1\rightarrow3)G_D\,\beta\,(1\rightarrow4)2_D$	(16)		G, 2, (3), (6)	G, 2, (3)	A, B, C	2.8
2 - 6	(17)		2, 6	2		

Key:
- G Galactose
- A 2, 3, 4, 6-tetra-o-methylgalactose
- B 2, 4, 6-tri-o-methylgalactose
- C 2, 3, 6-tri-o-methylgalactose
- 2 2-o-methylgalactose
- 6 6-o-methylgalactose

All the oligosaccharides obtained on partial hydrolysis of the polysaccharide, viz. (2), (3), (6), (7), (14), with the exception of (17), were obtained from the acetolysis. In addition several new oligosaccharides were characterised; significantly a large number contain (1→3) glycosidic linkages, this linkage being much more stable to acetolysis than partial hydrolysis. Methylation studies on the desulphated polysaccharide indicate the presence of equal quantities of (1→4) and (1→3) glycosidic linkages in the polymer. All the trisaccharides obtained were found to contain both these linkages, and that they probably occur in an alternating sequence is supported by the structure of the only isolated tetrasaccharide (oligosaccharide 10). An alternating sequence of α(1→3) and β(1→4) glycosidic linkages is well established in several sulphated polysaccharides obtained from Rhodophyceae species.[104] Oligosaccharides (8) and (9) have been obtained by Lawson and Rees[95] from an acetolysis of λ-carrageenan. They obtained oligosaccharide (9) in a crystalline form but were unable to crystallise oligosaccharide (8). The o-β-D-galactopyranosyl-(1→4)-o-α-D-galactopyranosyl-(1→3)-D-galactose (oligosaccharide 8) from Pachymenia carnosa crystallised readily from methanol, from a mixed fraction. A possible suggestion for this difference is that Rees et al obtained almost equal amounts of the two trisaccharides whereas in this case oligosaccharide (8) occurred to a much larger extent than oligosaccharide (9). Oligosaccharide (15) was not obtained pure, but only combined with oligosaccharide (13). The structure of oligosaccharide (15) was, thus, determined by difference, but the results obtained were unambiguous. The only oligosaccharides which have not been completely characterised are (4) and (17). Although the linkage of (4) has been determined the anomeric configuration cannot be ascertained. From the low optical rotation of the saccharide it would appear that one of the component monosaccharides occurs as the L-isomer,

but insufficient material was available for this to be verified. There is no doubt at all that oligosaccharides (4) and (6) are two distinct species. It was not possible to methylate a sample of oligosaccharide (17) due to the small amount of material obtained (4mg), but by comparison with other oligosaccharides containing 2- or 6-o-methylgalactose a (1→3) linkage is predicted. This is inconsistent with the low optical rotation of the saccharide, but the experimental value is suspect due to the small amount of material available.

Five of the oligosaccharides investigated have been isolated from other algal polysaccharides: Parolis and Nunn obtained oligosaccharide (2) from phyllymenan[84] and aeodan,[105] while oligosaccharides (1), (8) and (9) have been obtained from λ-carrageenan.[95, 106] A partial hydrolysis of phyllymenan[84] also yielded oligosaccharide (6).

From the oligosaccharides obtained it appears that, except for alternating β(1→4) and α(1→3) glycosidic linkages, there is no overall repeating pattern in the polysaccharide. However, it is possible to make certain generalisations: the 2-o-methylgalactose and 6-o-methylgalactose in all isolated oligosaccharides are linked glycosidically through positions 4 and 3, respectively, although the monomer to which they are glycosidically linked can vary (c.f. 6 and 14); galactose occurs linked through position 3 or 4 (oligosaccharides 1 and 2); the number of adjacent galactose units varies to a considerable extent, from at least four (oligosaccharide 10) to one (oligosaccharide 16).

The presence of pyruvic acid in several species of agar[107] and in the sulphated polysaccharide from Anatheca dentata[85] raises the possibility of its widespread distribution in algal polysaccharides. For this reason several polysaccharides were screened for pyruvic acid[108] and

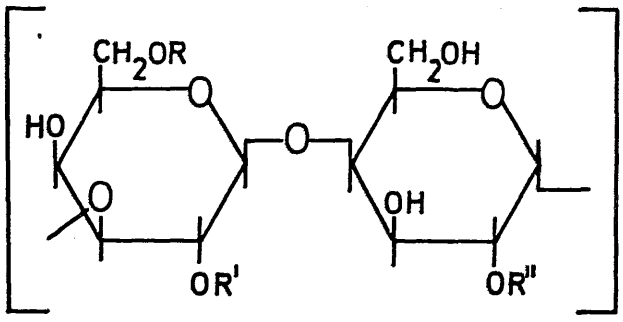
it was found present in the sulphated polysaccharides from three Grateloupiaceae species, viz. Phyllymenia cornea, Aeodes orbitosa and Aeodes ulvoidea. However, there is no evidence for the presence of pyruvic acid in Pachymenia carnosa.

The results obtained indicate that, at least in gross features, the sulphated polysaccharide obtained from Pachymenia carnosa is similar to to those obtained from other members of the Grateloupiaceae family.[18, 79, 84, 92, 100, 105] In all cases the major monomer is D-galactose, and this occurs in the polymer together with substituted galactoses. The nature of the substituent varies from polysaccharide to polysaccharide, for example, the major monomethyl sugar is 2-o-methyl-D-galactose in aeodan and 4-o-methyl-L-galactose in the sulphated polysaccharide from Aeodes ulvoidea. The polysaccharide from Grateloupia elliptica differs from the others investigated in that it contains appreciable amounts of L-galactose and 3, 6-anhydro-D-galactose, and traces of 2-o-methyl-L- and 4-o-methyl-D-galactose. The polysaccharides all contain large amounts of sulphate, most of which is alkali stable.

From methylation investigations, phyllymenan, aeodan and the polysaccharide of A. ulvoidea all have a backbone of (1→3) and (1→4) glycosidic linkages, although only in the polysaccharide from A. ulvoidea is an approximately 1:1 molar ratio of these units indicated; in the other two polysaccharides there is a predominance of (1→3) glycosidic linkages. There is no direct evidence of branching in these polysaccharides although it is possible that the polysaccharide from A. ulvoidea is branched to a small extent: all the 4-o-methyl-L-galactose occurs as non-reducing end-groups which implies either a short chain polysaccharide or branching.

The 2-o-methyl-D-galactose in this polymer, in aeodan and in the polysaccharide from P. carnosa appears unattacked by periodate and must, therefore, occur as (1→4) linked units in the polymer. No sulphated sugars have been isolated from any of these polysaccharides but methylation studies on native phyllymenan and aeodan have indicated the presence of galactose and/or 6-o-methylgalactose 2- and 4- sulphate, in both these polymers, and, in addition, galactose-2,6-disulphate from aeodan. An indication of the fine structures of aeodan and phyllymenan have been obtained from partial hydrolyses: 4-o-β-D-galactopyranosyl-D-galactose and 3-o-D-galactopyranosyl-D-galactose were isolated from aeodan, confirming the presence of (1→4) and (1→3) glycosidic linkages in the polymer; and four disaccharides were obtained from phyllymenan viz. 4-o-β-D-galactopyranosyl-D-galactose, 4-o-β-D-galactopyranosyl-2-o-methyl-D-galactose, 4-o-β-D-galactopyranosyl-L-galactose and a 6-o-methyl-D-galactopyranosyl-2-o-methyl-D-galactose, the isolation of a disaccharide containing L-galactose being the first indication that the polymer contains this monomer.

Phyllymenan is the only polysaccharide obtained from the Grateloupiaceae for which a partial structure has been suggested viz:

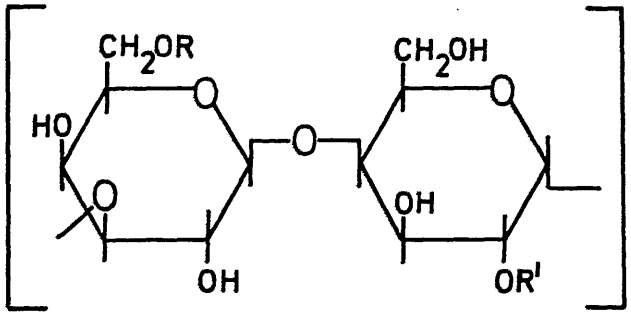


where R=H and less frequently CH_3; R′ = SO_4^{2-} and less frequently H; R′′ = CH_3 and less frequently H.

It is not possible to rigorously compare the four sulphated

polysaccharides isolated from Grateloupiaceae species as not sufficient is known about their individual fine structures.

From the results obtained it is evident that the polysaccharide from Pachymenia carnosa is complex, and has as its basic structure an alternating series of galactose or substituted galactose units linked α(1→3) and β(1→4), which may be branched but, if branches are present, they are readily removed with methanolic hydrogen chloride. At present it is not possible to propose a unique structure for this polysaccharide. However, the following partial structure is consistent with evidence obtained from studies on the neutral polymer.



where R = H or CH_3; R′ = H or CH_3

No attempt has been made to suggest the position of ester sulphate in this structure.

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