STRUCTURAL INVESTIGATIONS ON POLYSACCHARIDES OF THE CHLOROPHYCEAE

By P.G. JOHNSON, B.Sc.

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CHEMISTRY DEPARTMENT,
ROYAL HOLLOWAY COLLEGE,
(UNIVERSITY OF LONDON),
ENGLEFIELD GREEN,
SURREY.

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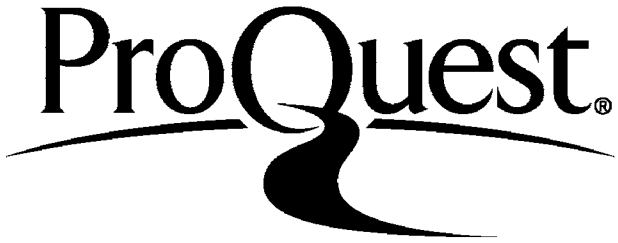

To my Parents

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ABSTRACT

Structural studies on the hot water-soluble starch-free polysaccharide extract from the green alga, *Cladophora rupestris*.

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1. Gel column chromatography, gel electrophoresis, and ultrafiltration, gave no significant fractionation of the above material into homopolymeric species.

2. Three sequential Smith's degradations of the polysaccharide and quantitative analysis by gas liquid chromatography of the hydrolysates of both the polymeric materials and fragments has established the mutual linkage of the individual sugar units; confirmed that all the xylose and some of the galactose units are present on the periphery of the molecule; and provided additional information on the overall structure of the macromolecule. A novel mild hydrolysis technique was developed. Gel column chromatography gave a measure of the degree of cleavage occurring in the polymeric materials.

3. Autohydrolysis resulted in the isolation and characterisation of disaccharides containing galactose and xylose. Modification of the technique gave sulphated oligomers comprising galactose and arabinose, a large proportion (60%) consisting of a mixture of sulphated pentasaccharides (galactose : arabinose 1:4). By alkali treatment, iono-

phoresis, methylation and periodate oxidation it was possible to deduce the overall structure of these oligomers.

4. Partial desulphation with methanolic hydrogen chloride gave a 63% recovery of polysaccharide in which the sulphate content was halved. The recovered polysaccharide, from which a considerable proportion of the galactose had been removed, was completely methylated and the derived methylated sugars characterised. The major structural feature is 1,4 linked arabinose, with xylose constituting the major end group.

The three methods of attack on this polysaccharide have provided complimentary results and from them the highly branched nature of the polymer is confirmed, the various linkages between sugars elucidated; and, for the first time, the presence of a repeating structure, comprising blocks of at least eight 1,4-linked arabinose units, some sulphated at C-3, linked together by single galactose residues, is established as a major feature of the polysaccharide.

Furthermore sequential Smith's degradation provides a simple technique for use in chemotaxonomic studies of this type of polysaccharide.

CONTENTS

GENERAL INTRODUCTION

"It is generally agreed that biological classification is an intellectual procedure which allows us to come to grips with, and organize the vast amount of information which we can perceive in the natural world. It involves the formation and description of taxonomic groups (species) and the grouping of these into a limited number of more inclusive groups (genera, families, orders etc.) based upon the degrees of similarity in respect of greater or lesser numbers of the characters possessed by the organisms concerned," so writes Heywood.[1]

There are however two distinct approaches to taxonomy, they are the empirical and the evolutionary or phylogenetic systems. The empirical system is the production of a classification based upon morphological characteristics (anatomical, reproductive) into which species may be fitted precisely. The phylogenetic system not only produces a well defined classification but in addition it seeks firstly to ensure that the various taxonomic levels are natural and secondly that they indicate the relationships of the various groups. This serves to separate the two systems. The empirical system considers a maximum number of equally emphasised characters and then, using mathematical correlation procedures, groups those with high correlation

figures together. This may well lead to both natural and artificial groups.

The phylogenetic system is based on the hypothesis that all species were formed by the process of organic evolution and that a classification based on features of evolutionary importance will produce the most accurate and natural system. This implies that characters must be weighted. Weighting is a process of selecting characters of particular importance and giving them more significance than the rest of the features of the material. Since the weighting procedure depends so much on insight and opinion, this is the major weakness inherent in this taxonomic system.

The problem is resolved partially by considering a larger number of variably weighted characters and comparing their correlation functions. This is the point at which the two systems become amalgamated into one, the mathematical approach of the empirical system and the weighting of the phylogenetic system. At the two defined extremes of taxonomy there are distinct differences in concept but between them the boundaries have lost much of their clarity. As a result the best type of classification is formed, which is still phylogenetic since it has the aim of understanding the evolutionary changes that have occurred but is probably

more accurate since it combines with this the mathematical approach of the empirical system.

The characters that were previously used for the systems of classification were mainly morphological with a few very obvious chemical features. At the present time, with the stimulation afforded by the greater amount of chemical studies on natural products, other characters are becoming available to the taxonomist. One major group of compounds to have been studied are the plant polysaccharides.

It is obviously very dangerous to base any system of classification on single characters; in fact it is required of any classification system that it should be based on several features. At the same time this does not imply that classifications based on single criterion are not useful; but it does mean that confirmation with other criteria is needed to minimise any tendency towards artificial groupings. With reference to the marine algae how do the polysaccharides help the taxonomist to group the species into the higher taxonomic levels ?

Of the major classical divisions of the algae only the polysaccharides of the Rhodophyceae (red algae) Phaeophyceae (brown algae) and Chlorophyceae (green algae) have been extensively studied. These fall into three major groups

namely; the reserve polymers; the structural polymers; and finally the acidic polymers whose function is largely a matter of conjecture. This last group will be dealt with first.

ACIDIC POLYMERS

This group of polymers includes the galactan sulphates, the polyuronides, and the sulphated polysaccharides containing a variety of sugar residues.

The Galactan sulphates

Galactan sulphates from many different species have been studied. They are essentially linear polymers of alternating 1,3- and 1,4-linked galactose units. In many instances this simple pattern is masked by two other features. Firstly, some of the 1,4-linked galactose units are present as the 3,6-anhydro-derivative, a change that can be brought about through loss of sulphate by both enzymic action or treatment with alkali, and secondly, many of the galactose units of both linkage types are sulphated or methylated at the various unsubstituted hydroxyl groups.

Three reasonably distinct types of polymer have been recognised, these are the agar-type, the porphyran-type and the carrageenan-type polysaccharide.

The agar-type polymer is in fact a mixture of two closely related polymers, agarose and agaropectin. Studies on the agarose fraction have shown that it is a linear polymer composed of alternating β-1,3-linked-D-galactopyranose and α-1,4-linked 3,6-anhydro-L-galactopyranose units, a conclusion based on the fact that nearly theoretical yields of the disaccharides, agarobiose, 4-O-β-D-galactopyranosyl-3,6-anhydro-L-galactose and neoagarobiose, O-3,6-anhydro-α-L-galactopyranosyl-(1→3)-D-galactose, have been isolated from methanolysates and enzymic hydrolysates respectively.[2,3] This polymer contains no ester sulphate and is the only algal galactan which is devoid of this feature.

The nature of the reducing and non-reducing end-group has been postulated as a result of two studies. Enzymic degradation, in which the disaccharide neo-agarobiose (O-3,6-anhydro-α-L-galactopyranosyl-(1→ 3)-galactose) and the corresponding tetraose have been isolated,[3] and neither free D-galactose nor free L-galactose could be detected, indicating perfect cleavage based on an anhydro non-reducing end group and a D-galactose reducing group. The second piece of evidence for this structure comes from methylation studies on the polymer in which 2,3,4,6-tetra-O-methyl-D-galactose has never been detected. The structure of this type of polymer seems reasonably well established except that

from different species of seaweed variable amounts of 6-O-methyl-D-galactose have been observed.[4]

GENERAL FORMULA

$$[\text{-3-D-Gal}(1\rightarrow 4)\text{-L Agal}(1]_x \longrightarrow [3)\text{-D-Gal}(1\rightarrow 4)\text{-L-Agal}(1]_y \longrightarrow$$

(6-OMe)

The structure of the second form, agaropectin, is less well understood. It is composed of D-galactose, 3,6-anhydro-L-galactose, ester sulphate, and D-glucuronic acid. In one case L-arabinose[5(a)] and pyruvic acid[5(b)] have also been isolated from hydrolysates of this fraction. The pyruvic acid has been shown to be part of the molecule in which it forms a 4,6-ketal with a galactose unit. In contrast the L-arabinose has not so far been shown to be part of the molecule with which it occurs.

Enzymic studies have intimated that agaropectin has some features in common with agarose since neo-agarobiose, -tetraose, and -hexaose, have been indicated as structural units as well as acidic components. Whether the glucuronic acid is part of the molecule remains uncertain and the position of the sulphate ester has yet to be established. It has been suggested that this molecule is the precursor of agarose.

The porphyran type polymers are also essentially linear molecules composed of α-(1→3)-linked-D-galactopyranose or

-6-O-methyl-D-galactopyranose residues alternating with L-galactopyranose-6-sulphate or 3,6-anhydro-L-galactopyranose residues linked β-(1→4).[6]

Enzymic studies on the polymer have yielded, D-galactose, 6-O-methyl-D-galactose, as well as the disaccharide neo-agarobiose, and the tetraose based on this unit. A tetraose, containing 1 mole of D-galactose, 1 mole of 6-O-methyl-D-galactose, and 2 moles of 3,6-anhydro-L-galactose, was also isolated in addition to some sulphated oligomers.[8]

Methylation of the alkaline-modified polymer gives a molecule with the same constitution as methylated agarose and partial methanolysis of this derivative gives a methylated form of agarobiose.[7]

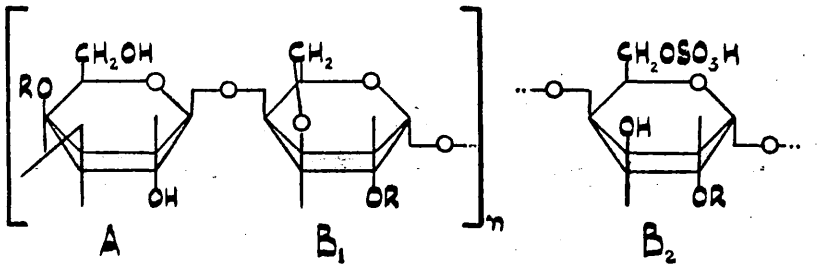
All this serves to indicate the high degree of similarity between the structure of this type of polymer and that of agarose, the major difference lying in the degree of sulphation of the C-6 of the L-galactose residues and the extent of methylation of the C-6 of the D-galactose units.

The final group of the galactan sulphates are the carrageenan-type polymers. These represent a very complex mixture, or family, of polysaccharides varying in the extent to which they are sulphated and the position of the sulphate ester groups. The monosaccharide is D-galactose, either as the normal residue or as its 3,6-anhydride, both of which may

be sulphated. The carrageenans are thus very distinct from the previous polymer-types since they are an all D-galactose system. A small amount of L-galactose has been reported in two samples,[9A,9B] and this may be of importance in deducing phylogenetic relationships.

The mixture of polymers may be fractionated by the addition of potassium chloride,[11A] into a κ-carrageenan, insoluble, and a λ-carrageenan, soluble, fraction. κ-carrageenan is an essentially linear polymer composed of D-galactose, 3,6-anhydro-D-galactose, and several differently situated ester sulphate groups. The linkage is an α-(1→3), β(1→4) alternating system which is heavily masked by the presence of the sulphate groups. The disaccharide, carrabiose, O-β-D-galactopyranosyl-(1→4)-3,6-anhydro-D-galactose, and neo-carrabiose sulphate O-α-3,6-anhydro-D-galactopyranosyl (1→3)-D-galactose-4-sulphate have been isolated, the former as the dithioacetal from mercaptolysis[12] and the latter by enzymic studies using κ-carrageenase from Pseudomonas carrageenovora.[13] The resistant residue from the enzymic study is a highly sulphated polymer which after alkali modification is again open to enzymic attack further indicating the presence of 6-sulphated entities. The 1,3-links are often associated with 4-sulphated units and the 1,4-linked-3,6-anhydro sugar residues are associated to some

extent with 2-linked sulphate.[14,15] The 1,4-linked-D-galactose units are sulphated at positions 6 or 2 and 6, hence representing the precursors of the 3,6-anhydro(1→ 4)-linked residues.



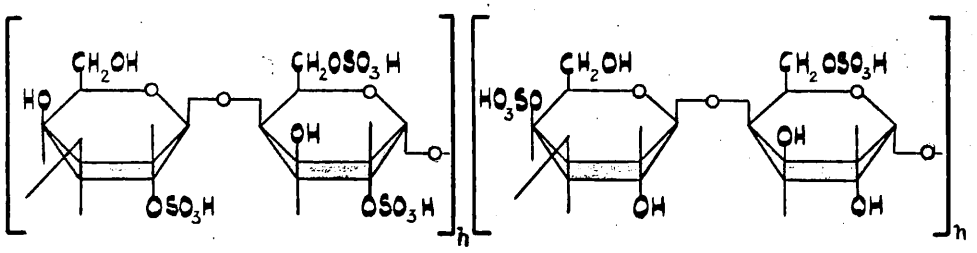
R = H or SO_3^-, B_1 and B_2 are alternatives.

GENERAL FORMULA OF κ-CARRAGEENAN

The second fraction λ-carrageenan represents an even more complex system. This is the portion left in the supernatant after potassium chloride fractionation. The material is very highly sulphated and contains D-galactose, a smaller proportion of 3,6-anhydro-D-galactose and in some species a very small proportion of L-galactose.[9A,9B] Rees[15] has indicated that in the samples he has studied there is no L-galactose present. The material may be further fractionated in alcohol to give a polymer based entirely on D-galactose.[11b] This polymer conforms to the alternating 1,3-, 1,4-, linkage system which is again strongly masked by the presence of sulphate ester groups. Some of the sulphate ester can be

removed by treatment with alkali causing an equivalent increase in the 3,6-anhydro derivative, indicating the presence of sulphate on C_3 or C_6 of some galactose units. This alkali-modified polymer can be further fractionated in potassium chloride solution[16] giving an insoluble fraction μ-carrageenan, which contains all the 4-sulphate associated with the 1,3-linked D-galactose units and much of the 3,6-anhydro-D-galactose as unsulphated units, which were probably 6-sulphated in the unmodified form.

The soluble component, λ-carrageenan, in this modified form, contains all the 2-sulphated 3-linked-D-galactose units and a high proportion of the 2-sulphated 4-linked-3,6-anhydro galactose units, again in the unmodified form the anhydro units are present as the 6 sulphate.



λ-CARRAGEEN TYPE UNIT μ-CARRAGEEN TYPE UNIT

Finally in this group of polymers there exist polysaccharide extracts from the Grateloupiacean species Aeodes orbitosa[10a,17] and

Phyllymenia cornea which as yet have not been shown to be of the linear alternating 1,3-, 1,4-linkage constitution but are galactans with a high sulphate content and D-galactose as the major sugar residue. Aeodean however contains a small quantity of glycerol but both polymers contain 2-O-methyl-D-galactose, 6-O-methyl-D-galactose and 4-O-methyl-L-galactose. A trace of xylose has also been observed in hydrolysates of the extracts. The presence of 1,3-linked-2-sulphated, 4-sulphated, and 2,6-disulphated, units have been indicated in the case of Aeodean. Some of the 1,4-linked units are 6-sulphated but many are sulphate-free.[17] The presence of β-1,4-links has been indicated by the isolation of 4-O-β-D-galactopyranosyl-D-galactose, from partial acid hydrolysates for both polymers. Using the same technique a 1,3-linked galactobiose was also isolated from Aeodean but the type of anomeric link was not determined.[10] The molecules, Aeodean and Phyllymenan, appear to contain unequal numbers of 1,3- and 1,4-links and hence, although no absolute structure can be assigned to them, it would seem probable that the materials depart from the strictly alternating 1,3- 1,4-pattern.

From this brief account it is clear that a whole family of galactans, based, with the exception of the members of the Grateloupiaceae, on the alternating linkage system, but

differing in the degree of modification and substitution, are metabolised by a group of algae.

In the taxonomic field these polymers are very important since they all occur in a single group, the Rhodophyceae, which has been defined by other features. They confirm the distinctness of this group and imply that it does indeed represent a natural taxonomic level. However these polysaccharides may have further phylogenetic significance since, as is very obvious in the case of the agar-type and porphyran-type polymers, a gradual merging of properties has occurred. If one considers the normally accepted classification of the species examined and the type of polymer they contain, a pattern becomes apparent. (see TABLE I).

This pattern is of interest to the taxonomist since it suggests certain connections between these groups. It may very well indicate, with reinforcement from other features, a true phylogenetic linkage. How a change from a D; L-alternating system to an all D-system (or vice versa) could have occurred is unknown but the situation in both Chondrus and Aeodes is very intriguing, whether in some algae a galactan exists with variable contents of both D- and

CARRAGEENAN CONTAINING	AGAR CONTAINING	PORPHYRAN CONTAINING
O-GIGARTINALES	O-GIGARTINALES	
F-GIGARTINACEAE	F-GRACILLARIACEAE	
G-CHONDRUS	G-GRACILLARIA	
G-GIGARTINA	F-PHYLLOPHORACEAE	
F-FURCELLARIACEAE	G-AHNFELTIA	
G-FURCELLARIA		
	O-NEMALIONALES	
O-CRYPTONEMIALES	F-GELIDIACEAE	
F-GRATELOUPIACEAE	G-GELIDIUM	
G-AEODES	G-PTEROCLADIA	
	O-CERAMIALES	O-CERAMIALES
	F-CERAMIACEAE	F-RHODOMELACEAE
	G-CERAMIUM	G-LAURENTIA
		O-BANGIALES
		F-BANGIACEAE
		G-PORPHYRA
		G-BANGIA

KEY

O = ORDER

F = FAMILY

G = GENUS

TABLE I

L-galactose and in which both sugars are present in more than trace quantities but less than equal quantities remains to be determined.

Alginic Acid

The second group of acidic polymers are the polyuronides known collectively as Alginic Acid. This type of polysaccharide is composed entirely of uronic acid residues. Initial work[18] indicated the presence of only one type of uronic acid in the material, namely D-mannuronic acid. However as a result of work by Fischer and Dörfel,[19] a second type of residue, L-guluronic acid, was discovered.

The polysaccharide extract can now be fractionated into guluronic acid—rich, mannuronic acid-rich, and mannuronic/guluronic acid mixed polymers. Addition of potassium chloride to solutions of the sodium salt of the acid results in the precipitation of the mannuronic acid-rich fraction.[20] Addition of potassium chloride and manganous sulphate to the same alginate salt causes the precipitation of the guluronic acid-rich fraction.[21] Complete separation, either of a pure mannuronic or guluronic acid containing polymer, has never been accomplished except by extensive acid degradation which can give rise to pure single acid polymers of around 25 units.[22]

Structural studies,[23-27] have shown that both acids are

1,4-linked in the form of a linear molecule. Partial acid hydrolysis of a carboxyl reduced molecule[28] has led to the isolation and characterisation of β-1,4-linked mannobiose which, with the high negative rotation of the original extract, implies that much of the molecule is composed of β-linked residues. This procedure also led to the isolation of a mannosyl-gulose, of undetermined linkage, proving that the material is indeed heteropolymeric and not a mixture of two homopolymers. At the present time the molecule is believed to be composed of blocks of mannuronic, or of guluronic, acid residues, about 20-30 units long separated by areas composed of both acid residues, to a large extent in an alternating pattern.

The importance of this polymer to the taxonomist is that it is confined to one group of algae, the Phaeophyceae, and hence gives further evidence of the discrete and natural form of this group. The fine structure might be expected to indicate some further points of value to the taxonomist. However, the most obvious feature, the mannuronic/guluronic acid ratio (M/G), fails to do this. Several groups of workers have shown,[19,29,30] that, not only do the ratios vary from individual to individual with change in season but they also vary according to the tissue examined.[30] This latter

variation is more stable as a relative term, for instance the M/G of Alginic Acid in the medulla is always higher than it is in the cortex tissue in Laminaria hyperborea. The present state of knowledge does not permit any further taxonomic conclusions but it may well be that further studies will produce some finer details of structure which may have significance. One further polymer which also helps define the Phaeophyceae as a natural group should be mentioned, this is Fucoidin.

Fucoidin is a polymer whose absolute structure has not yet been completely diagnosed. It contains L-fucose as the major neutral sugar with traces of D-galactose and D-xylose and in addition a high ester sulphate content. Structural studies on the polymer, which have included methylation,[31] alkali lability,[31] and acetolysis,[33,34] have indicated that the 1,2 link is present in high proportion, and that much of the sulphate is at C-4 of the pyranose unit.[35] Some 1,3- and 1,4-linkages have also been indicated,[34] and these are believed to be associated with branch points. Whether the trace sugars are really linked to the fucose units in the polymer remains uncertain but with further investigation it is hoped that this problem will be clarified.

Although Fucoidins from several species of the Phaeophyceae have been studied, for example Fucus vesiculosus, much remains to be determined of the macro-molecular structure, hence as yet little comment can be made about their taxonomic significance. Another similar fucose containing polymer found in thePhaeophyceae which is also of uncertain taxonomic merit is a polymer normally found contaminating alginic acid in Ascophyllum nodosum known either as Ascophyllan or as a glucuronoxylofucan. The material contains the sugars L-fucose, D-xylose, and D-glucuronic acid. There is a great deal of controversy about the gross structure of the polymer. Haug[61] has carried out mild acid hydrolysis and the degraded polymer recovered was composed almost entirely of glucuronic acid from which he deduced that the material had a glucuronic acid backbone. However Percival[62] in other partial acid hydrolysis studies has obtained evidence which implies that the glucuronic acid is not so disposed. The two materials were isolated differently and they contain somewhat different proportions of the sugars. It is probable that a whole family of such polymers are synthesised by the individual members of the Phaeophyceae. However no taxonomic significance can be deduced.

ACIDIC POLYSACCHARIDES CONTAINING MIXED SUGARS

The third and final group of acidic polymers are the sulphated polymers in which mixtures of sugars are found. These are best separated into two groups. The first group consists of sulphated materials containing D-galactose, L-arabinose, and D-xylose as the major sugar residues. The second group also sulphated contains the sugar units L-rhamnose, D-xylose and D-glucuronic acid.

The former group will only be discussed in a very general way since this type of polymer represents the subject of this thesis and hence will be discussed in greater detail at a later stage. These materials invariably have a high content of D-galactose but the pentoses seem very variable, in some cases L-arabinose being the major pentose constituent and in others D-xylose, trace quantities of rhamnose have also been observed. The sulphate contents seem less varied, and in all of them it seems impossible to remove the last traces of protein. All attempts to fractionate the materials into homopolymers have so far failed, as has the search for evidence in favour of their heteropolymeric nature.

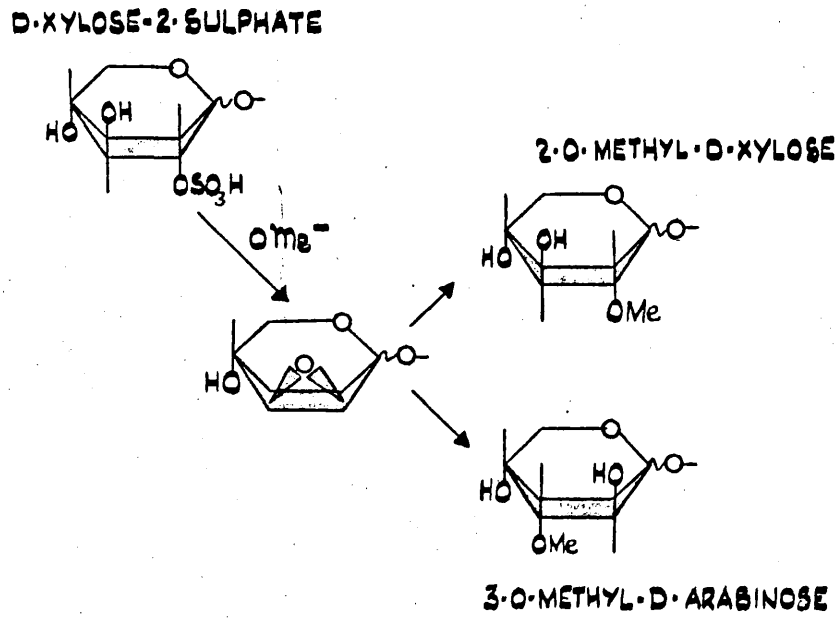
The second group of polysaccharides are heteropolymeric and contain L-rhamnose as the major neutral sugar with smaller

quantities of D-xylose and traces of D-glucose. The proportion of D-glucuronic acid present seems relatively constant (ca. 20%) but the content of ester sulphate is variable.

Partial acid hydrolysis has indicated that the extracts are heteropolymeric, since in all cases studied an aldobiouronic acid, 4-O-β-D-~~glucopyranosyl~~glucopyranuronosyl-L-rhamnose, has been isolated and characterised.[36,37] Methylation of the polymers,[36,38] containing free carboxyl groups and high sulphate ester contents, has proved difficult but in all cases has confirmed the presence of 1,4-linked-L-rhamnose and also indicated 1,4-linked, as well as end group, D-xylose.

More detailed and extensive studies have been carried out on only one of the polymers, that from Ulva lactuca. The site of ester sulphate has been deduced from several sources of experimental evidence. Periodate oxidation of the desulphated polymer at 0°C (which specifically cleaves cis-diols) has led to a marked decrease in rhamnose in the derived polyalcohol indicating that the 2 or 3 position in rhamnose was previously sulphated. Since this sulphate ester was alkali stable and gave infra-red peaks consistent with an axial sulphate group it was assigned to the C-2 of L-rhamnose which in the 1C conformation is axial. Ester sulphate is also found on the C-2 of some of the xylose units and was assigned this position because of its alkali

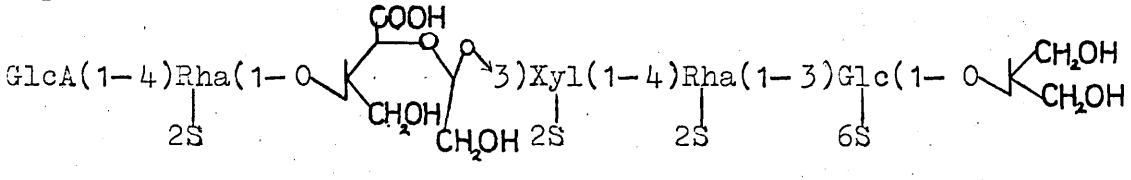
lability and the products formed by treatment with methoxide ion.[39]



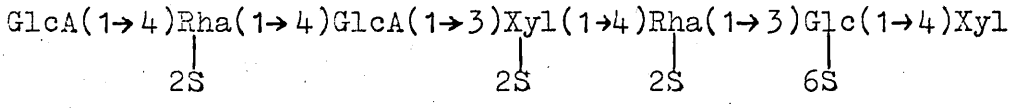
Partial acid hydrolysis studies[40] on the initial polysaccharide have indicated that glucuronosyl-(1→3)-xylose and glucuronosyl-(1→4)-xylose are structural units in the macromolecule and in the desulphated carboxyl reduced form, the oligosaccharide rhamnosyl-(1→4)-xylosyl-(1→3)-glucose was shown to be present (N.B. glucose = reduced form of glucuronic acid).

Smith's degradation[41] studies on the initial polymer led

to the characterisation of a sulphated hepta-saccharide which represents a major repeating unit and is believed to represent the backbone of the molecule.



The polymer is thus considered to be based on the type of unit:



GlcA = D-glucuronic acid, Rha = L-rhamnose, Xyl = D-xylose, Glc = D-glucose, S = sulphate ester.

The absolute order of sugar units is still uncertain.

Although the results of structural studies on the polymer from Ulva lactuca cannot be applied rigidly to the other extracts it is fair to assume that they are probably based on a similar constitution.

The species containing either of the two types of extract are all found in one taxonomic group, the Chlorophyceae. These polymers neither imply that this grouping is natural or unnatural, what they do suggest is that the group may be polyphyletic (that is, the group has several ancestoral types) or that a very fundamental split occurred at an early

stage in the group's development. The presence of xylose and rhamnose in all the polymers gives weight to the latter suggestion. Until more is known, especially in the case of the galactose containing group of polymers, only these brief, and clearly tentative suggestions can be made.

FOOD RESERVES

The second major class of algal polysaccharides, the "food reserves", are composed of three types of glucan, these are laminaran, floridean starch, and starch.

Laminaran is a polymer entirely composed of D-glucose residues linked 1,3 or 1,6, the latter representing only a minor percentage. The linkage is β since, firstly, the polymer has a small negative $[\alpha]_D$ [43] and, secondly, the disaccharide laminaribiose (3-O-β-D-glucopyranosyl-D-glucose) has been obtained from partial acid hydrolysis studies.[44] The position of the 1,6- link remains uncertain since evidence exists to suggest that it occurs at branch points and/or inter-chain positions.

In some genera e.g. Laminaria, some of the chains are terminated at the potential reducing end group with mannitol,[42] the remaining chains having the normal glucose end group. In other species[45,46] e.g. Eisenia bicyclis and Ishige okamurai, this feature has not been observed but a much higher

proportion of 1,6-linkages does occur.

In the algae this polymer has been observed in the Phaeophyceae, the Chrysophyceae (grass green algae), the Euglenophyceae and in two isolated cases in the Chlorophyceae. The material is also widespread in nature having been observed in some fungal species, e.g. Yeast, in lichens (which have an algal component) and in the higher plants.[47] Since the polymer, or similar polymer , is so widespread it represents a poor diagnostic for a particular group of plants, however the fine structure may well be a very useful tool in the classification of the Phaeophycean orders, since, variability seems to occur in the fine details in different species.

The second food storage polymer, floridean starch, is also comprised completely of D-glucose residues. It resembles the amylopectin portion of land plant starches in that it is composed of a core of α-1,4-glucosyl-links with branching through α-1,6-links. The presence of a small portion of α-1,3-links has been shown experimentally[48] in the extract from Dilsea edulis and this may serve to distinguish it from amylopectin, as does the fact that the average chain length (13-15 units) is much smaller in floridean starch than in amylopectin.[49] This distinct group of polymers is found in one division only, the Rhodophyceae,

it thus serves to indicate once more the naturalness of this group.

The final group of storage products are the "true starches". These materials are composed of two polymers, a linear molecule containing α-1,4-linked D-glucose residues, amylose, and a highly branched molecule, amylopectin, containing both α-1,4- and α-1,6-linked D-glucose units.[50] Both these polymers are very similar to the land plant starches but are distinguished by their smaller average size.[50]

These starches, characteristic of higher plants, are confined to one group of algae, the Chlorophyceae, and hence imply the unity of this class, whether divided at an early stage or not remaining uncertain (cf the acidic polymers containing mixed sugars). It is of interest to note the similarity between the starch-type polysaccharides from the Rhodophyceae, the Chlorophyceae, and the higher plants. Whether this can be interpreted in a phylogenetic sense remains to be seen but in time it may well be shown to be correct.

STRUCTURAL POLYMERS

The final group of polymers are the structural polysaccharides. There are three categories of these, the xylans, the mannans, and the glucans.[51]

The xylans seen in the algae are of two linear types, they are either comprised entirely of β-1,3-linked D-xylose units as in the Chlorophyceaen species, such as Caulerpa or of β-1,3-, β-1,4-linked D-xylose units arranged in random fashion as is seen in the Rhodophyceae species, such as Rhodymenia palmata. This latter group of polymers has never been fractionated into a homo β-1,4- or β-1,3-linked polysaccharide and all the evidence points to a heteropolymer.

The mannans so far investigated are all composed of D-mannose units linked β-1,4 in linear polymers. In some instances a small proportion of D-glucose is present and has so far defied separation and in one case a true gluco-mannan has been found.[53] These polymers are restricted generally to the Rhodophyceae and Chlorophyceae.

The glucan polymer is better known as Cellulose. In all cases examined the physical and chemical evidence has indicated that this represents a true β-1,4-linked linear polymer of D-glucose. In certain cases the material is always encrusted with "hemicellulose" and this has somewhat obscured the detailed structure.

The main point of taxonomic interest of these structural polymers is at the very lowest levels, the specie and genus. As a result of their widespread and disjointed abundance

they are of little use to the taxonomist at the higher levels.

At the specific and generic levels they, however, serve to define with the aid of many other features the existence of a true specie or genus. They however seem unable to define phylogenetic links.

The importance of these polymeric materials have been indicated, in some cases they are discrete and thus increase the evidence in favour of the naturalness of certain algal divisions. They have indicated certain links which further taxonomic study may confirm as truly phylogenetic. Finally they have given, in some cases, useful information at the specific and generic level of taxonomy.

However, what is the present state of classification, what evolutionary links are now accepted and what further part can polysaccharide analysis play in the elucidation of further facts ?

The Danish Botanist Christensen[52] (1962) has suggested a classification based on phylogeny and also provided a diagrammatic representation of the evolution of the algae.

The classification is as follows (see TABLE II)

PROCARYOTA (NON-NUCLEATED)

DIVISION CYANOPHYTA

CLASS CYANOPHYCEAE

EUCARYOTA (NUCLEATED)

ACONTA (NO FLAGELLA)

DIVISION RHODOPHYTA

SUPER CLASS RHODOPHYCEAE

SUB CLASS BANGIOPHYCIDAE

SUB CLASS FLORIDIOPHYCIDAE

CONTOPHORA (FLAGELLATED)

DIVISION CHROMOPHYTA (CAROTENOIDS PREDOMINATE, CHLOROPHYLL b ABS.)

CLASS CRYPTOPHYCEAE
" DINOPHYCEAE
" RHAPHIDOPHYCEAE
" CHRYSOPHYCEAE
" HAPTOPHYCEAE
" CRASPEDOPHYCEAE
" BACILLARIOPHYCEAE
" XANTHOPHYCEAE
" PHAEOPHYCEAE

DIVISION CHLOROPHYTA (CHLOROPHYLLS PREDOMINATE, CHLOROPHYLL b PRESENT)

CLASS EUGLENOPHYCEAE
" LOXOPHYCEAE
" PRASINOPHYCEAE
" CHLOROPHYCEAE

ALGAL CLASSIFICATION (CHRISTENSEN 1962)

TABLE II

ALGAL PHYLOGENY

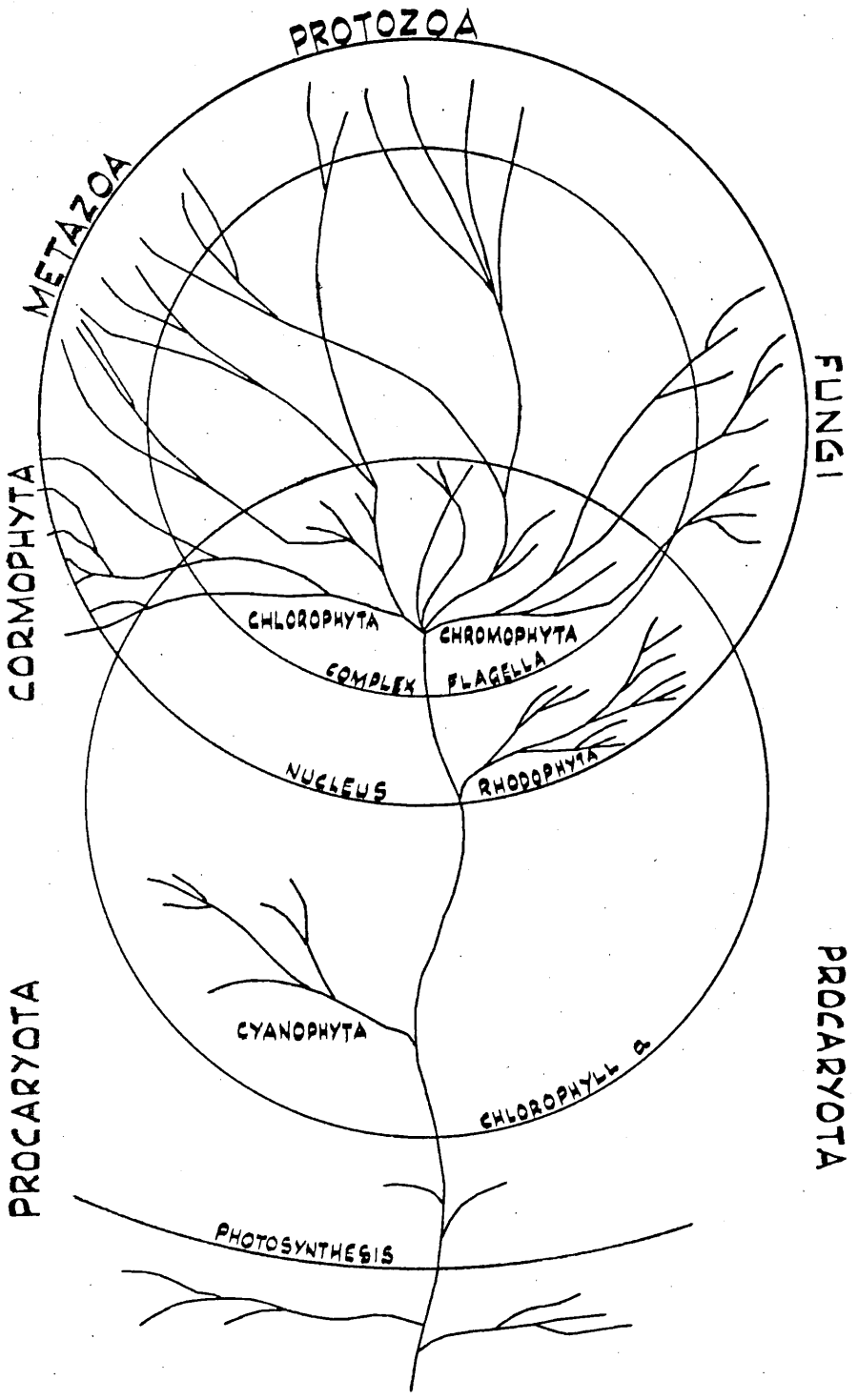


FIGURE 1

The three characters used to indicate the phylogenetic affiliations of the various groups of the algae are the presence or absence of, firstly, a true nuclear structure, secondly, a complex flagella (that is one having the normal 9+2 arrangement of fibrils), and thirdly, chlorophyll b. They are weighted in importance in that order. The classification is not based on a single character but on an amalgum of several, however, the problem still remains, how significant are the chosen characters?

In this particular classification the first and third characters are not really to be argued with. The problem is the second character, the presence or absence of a true flagella. No evidence exists to indicate that the ancestors of the Rhodophyceae did not have flagella which at some stage in their evolution they lost. The major biological arguments used as evidence in favour of the naturalness of this division are that, firstly, the flagella represents a stable system which is normally retained at some stage in the life cycle in the plant kingdom, and secondly, that it is also absent in the primitive Cyanophyceae. The weight of evidence probably favours this division but it is obviously not as precise as is required.

The fundamental reason why classification has to be used as a guide to the probable evolution of the Algae is that there is no fossil record of any magnitude for this group. Hence the utmost care is necessary when forming such a system. The system of Christensen must be viewed in the sense of a guide to further taxonomists, a pointer to a more accurate classification of the future.

A study of some of the polysaccharides of a limited number of groups has indicated that some of them are in agreement and hence add weight to the concept of the naturalness of certain taxonomic levels. The groups studied have been those of industrial importance (present and future) and thus represent a restricted category of algae. The study needs to be expanded to a much larger number of the groups and the results examined to see if any pattern is obtained which could be confirmed by a large number of other features. All the taxonomists tools must be used in parallel but not equally so.

The polysaccharides of the algae may be very useful in indicating phylogenetic links but they are more likely to be the bloodhounds of the taxonomeric world rather than the fundamental tool of classification.

INTRODUCTION TO THE HOT WATER SOLUBLE POLYSACCHARIDES OF THE CAULERPALES AND CLADOPHORALES

The hot water soluble polysaccharides of the Caulerpales and Cladophorales have been examined to a variable extent for the genera, *Caulerpa*,[53] *Codium*,[54] *Chaetomorpha*,[55] and *Cladophora*,[56] and they have been found to have some common general features.

They all have a comparatively high protein content which has defied complete removal. They all contain the monomers D-galactose, L-arabinose, D-xylose, and L-rhamnose, but have never been proved to be heteropolymeric species. The presence of D-glucose in the *Cladophora* and *Chaetomorpha* extracts is the result of contamination by glucans.

In the case of *Caulerpa* little more is known except that it is anomalous in containing a high proportion of D-mannose whether as a contaminant derived from a cell-wall mannan, or not, being uncertain. It is believed that at least some of the xylose is also present as a result of contamination by the low molecular weight fraction of a xylan known to be present.[57]

More work of structural significance has been carried out on the remaining three genera. The polysaccharides have, similar general physical properties (i.e. mucilages) with $[\alpha]_D$ + 45° - + 70°, similar sulphate contents (15-20%), and arabinose as a major constituent. The sulphate ester site

has been deduced from alkaline hydrolysis studies and from characterisation of sulphated fragments during partial acid hydrolysis studies.

In Codium, alkaline hydrolysis[54] caused a reduction in total sulphate content, and a concomitant formation of 3,6-anhydro-galactose was indicated, suggesting 3- or 6-sulphated galactose units. Traces of monomethyl pentose were also observed when the reagent sodium methoxide was used indicating the presence of a sulphated pentose. In Chaetomorpha and Cladophora[55] alkaline desulphation resulted in an increase in xylose content implying the presence of 2- or 3-sulphated arabinose. There was, however, no indication that 3,6-anhydro-galactose was formed in the reaction. Alkaline hydrolysis with sodium methoxide in the case of Cladophora, led to the isolation of 3-O-methyl L-arabinose and 2-O-methyl-L-xylose (cf. p.30) indicating arabinose sulphated in the 3 position. Similar results were indicated in the case of Chaetomorpha from paper and gas liquid chromatography.

Partial acid hydrolysis resulted in the isolation of galactose-6-sulphate (probably alkali stable as a result of being 1-3 linked) and arabinose-3-sulphate from Cladophora, with similar results indicated analytically in Chaetomorpha. Parallel studies on Codium resulted in the isolation of galactose 4- and 6-sulphates, but no arabinose sulphate was

observed even though the alkaline desulphation results were interpreted as indicating its presence. The oligomeric materials in these hydrolysates were also examined, 1→6- and 1→3-linked galactobioses and 1→4/5-linked arabinobiose were isolated from Cladophora and indicated in Chaetomorpha. The 1→3-linked galactobiose was also isolated from the Codium hydrolysate as well as a 1→3-linked arabinobiose.

More extensive studies have been carried out on the extract from Cladophora rupestris. These include methylation studies and a Barry degradation. The methylation, though incomplete as a result of the high sulphate content, indicated a high percentage of end-group xylose, some galactose in a similar position, 1→4/5-linked xylose, 1→6-linked galactose, 1→4/5-linked arabinose, and galactofuranose linked 1→6. The lower methylated monomers indicated 3,6-linked or sulphated galactose units, 2, 4/5-and 3, 4/5-linked or sulphated arabinose units. The relatively high percentage of monomethylgalactose, galactose and arabinose, probably resulted from undermethylation although the presence of some disulphated units may also be responsible for a portion of these features.

The Barry degradation,[58] periodate oxidation and cleavage of periodate oxidised units with acid phenylhydrazine,

results in the gradual erosion of the molecule to reveal the inner core. The results from that experiment[59] after successive degradations imply that the core of the molecule contained galactose arabinose and rhamnose (1:1:0.5). It also confirmed the methylation evidence that all the xylose was linked in such a way as to be periodate vulnerable since after the first oxidation it had all disappeared. A large drop in the relative galactose content in the first oxidation was taken to imply that much of this sugar was peripherally located.

	CODIUM	CHAETOMORPHA	CLADOPHORA
SUGAR CONTENT			
GALACTOSE	2	1.5	3
ARABINOSE	2	3.7	3.5
XYLOSE	1	1	1
% PROTEIN	25	19	8
$[\alpha]_D$	$+46^\circ$	$+66^\circ$	$+69^\circ$
SO_4 CONTENT	20	15	15
SITE OF ESTER	GAL 4 S GAL 6 S PENTOSE S	GAL 6 S ARAB 3 S	GAL 6 S ARAB 3 S
OLIGOMERS IDENTIFIED	GAL(1→ 3)GAL ARAB(1→ 3)ARAB	GAL(1→ 3)GAL GAL(1→ 6)GAL ARAB(1→ 4/5)ARAB	GAL(1→ 3)GAL GAL(1→ 6)GAL ARAB(1→ 4/5)ARAB

TABLE III

The results of these experiments have indicated a certain general similarity between the extracts of the Caulerpales and Cladophorales, (see TABLE III) however, if this type of component is to be used for any taxonomic purpose the structure must be known with a minimum amount of dubiety. The outstanding problems about these extracts which remain as yet unanswered, are, firstly, are they truly heteropolymeric or are they mixtures of homopolymers, secondly, the nature of the linkages between the three major monosaccharides, and thirdly, a firm idea of the form of the gross molecule. The purpose of this thesis is to see if some of the more modern techniques in organic chemistry can be applied to obtain such structural information, techniques, which, when applied to similar extracts from other species, could result in their structures being adequately defined.

The more modern structural methods applied in this study were

(a) Smith's degradation,

(b) Fragmentation studies using autohydrolysis,

(c) Desulphation followed by methylation,

and the major new analytical techniques used were

(1) gas liquid chromatography,

(2) agarose gel chromatography,

the former (1) representing the most powerful single chromatographic method so far devised for organic molecules with high vapour pressures.

E X P E R I M E N T A L

GENERAL METHODS

G.M. 1 EVAPORATION OF SOLUTIONS

Solutions were evaporated in vacuo using a Buchi rotary evaporator at 30°C unless otherwise specified.

G.M. 2 NITROGEN DETERMINATIONS

Nitrogen determinations were carried out by Alfred Bernhardt Laboratories.

G.M. 3 SPECIFIC ROTATIONS

All specific rotations were measured in aqueous solution at 20°C unless otherwise stated.

G.M. 4 ANALYSIS FOR PERIODATE

The spectrophotometric method of Aspinall and Ferrier[60] was used to measure the reduction of periodate in all oxidations unless otherwise stated.

G.M. 5 CARBOHYDRATE CONTENT

Carbohydrate content was estimated using the method of Dubois et al.[64]

G.M. 6 DEGREE OF POLYMERISATION (D.P.)

This was determined according to the method of Timell,[65] a modification of the method of Peat.[66]

For oligomers containing mixed sugar residues with D.P. 2 or 3 carbohydrate analyses were converted to weights

of sugar by referring to the relevant mixed sugar standard calibration curve. For oligmers of higher D.P. containing a higher proportion of one sugar this was not found to be necessary.

The D.P. was calculated according to the formulation:-

$DP = \frac{Q}{Q-1}$ where Q = ratio of non-reduced to reduced value of intensity measurement, or, carbohydrate content.

G.M. 7 ANALYSIS FOR SULPHATE

Sulphate content was estimated using the method of Jones and Letham.[67]

The samples to be analysed (ca. 10 mg.) were digested by heating for 16 hours at 100°C with Analar concentrated nitric acid (2 ml) in a sealed tube. A few grains of sodium chloride were also added. The resulting solution was evaporated to dryness washed with Analar concentrated hydrochloric acid and evaporated (x 2). The tube containing the residue was placed in an oven at 120°C for 2 hours. The residue was cooled and dissolved in 10-20 ml of water and an aliquot (1 ml) withdrawn for analysis. Results were expressed as percentage based on carbohydrate content, unless otherwise specified.

G.M. 8 ACID HYDROLYSIS

(a) Formic Acid

Carbohydrate (2-10 mg.) was dissolved in 90% formic acid (1-2 ml) in a 15 ml hard glass tube. Solid carbon dioxide (ca. 50-100 mg.) was added to saturate the atmosphere with this gas and the tube sealed after the solid CO_2 had evaporated. The tube was heated for 6 hours at 100°C after which it was opened and the solution was diluted (x 4) and heated unsealed for a further hour. This solution was then evaporated to small bulk, treated with methanol (x 2) and evaporated to dryness.

(b) Sulphuric acid

(i) Carbohydrate (1-2 mg.) was dissolved in 2N-sulphuric acid (1 ml) and the vessel sealed, and heated at 100°C for 6 hours after which it was cooled. This solution was extracted (x 3) with 5% di-N-octylmethylamine dissolved in chloroform and the extract discarded. The aqueous layer was washed to neutrality with chloroform. The aqueous layer was evaporated.

(ii) Carbohydrate (5-10 mg.) was dissolved in 2N-sulphuric acid (4-5 ml) and the tube sealed as before. The solution was heated at 100°C for 6 hours after which it was cooled and neutralised with Analar barium carbonate. After filtering out the solid the filtrate was evaporated to dryness.

G.M. 9 METHYLATION AND METHANOLYSIS

The methylation procedure adopted for the sulphated and unsulphated oligomers was twice via Kuhn's procedure (Perila and Bishop modification)[68] and once via Purdie's procedure.[69]

Methanolysis was carried out on the fully methylated oligomers by refluxing with 3.5% methanolic hydrogen chloride (4 ml) for 6-8 hours under anhydrous conditions.

G.M. 10 METHOXYL CONTENT

Methoxyl contents were determined by Zeisel's method,[70] apart from the methylated fractions (p.118) which were determined by Alfred Bernhardt Laboratories.

G.M. 11 DEMETHYLATION

Demethylation was accomplished using the method of Bonner et al.[71]

G.M. 12 PAPER CHROMATOGRAPHY

Analytical paper chromatography was carried out using Whatman No.1 paper. Preparative paper chromatography was carried out using Whatman No.1, No.3 and No.17 papers all of which were previously washed with distilled water or the solvent to be used for elution. Whenever No.17 paper was used, a wick consisting of No.3 paper was attached, with the aid of staples, according to the method of Frush,[72] components were detected by spraying side and centre strips or by the

imprint method of Frush.[72]

All paper chromatography was carried out using the descending elution technique. The solvents used were as follows:-

A Butanol : Pyridine : Water; 6:4:3

B Ethyl Acetate : Acetic Acid : Formic Acid : Water; 18:3:1:4

C Ethyl Acetate : Pyridine : Water; 10:4:3

D Ethyl Methyl Ketone half saturated with water, containing 1% ammonia.

E Butanol : Ethanol : Water; 40:11:19

G Butanol : Ethanol : Water; 40:11:19 with 1% ammonia

H Tate and Lyle (Benzene (1): Butanol (5): Pyridine (3): Water (3) - Upper Phase)

I Ethyl Methyl Ketone half saturated with water, saturated with Boric Acid.

R_{GAL} VALUES

The R_{GAL} value is quoted when the mobility of a component was expressed relative to the mobility of galactose.

$$\text{Hence } R_{GAL} = \frac{\text{DISTANCE MOVED BY COMPONENT}}{\text{" " " GALACTOSE ON THE SAME CHROMATOGRAM}}$$

R_G VALUE

The R_G value is quoted when the mobility of a component is expressed relative to that of 2,3,4,6-tetra-O-methylglucose

$$\text{Hence } R_G = \frac{\text{DISTANCE MOVED BY COMPONENT}}{\text{" " " TETRA-O-METHYLGLUCOSE}}$$

SPRAY REAGENTS

(i) Silver Nitrate[73]

(ii) Aniline oxalate

The air dried papers were sprayed with a saturated 50% aqueous ethanolic solution of aniline oxalate. The papers were developed at 105°C for 5 minutes.

(iii) p Anisidine[74]

(iv) Periodate/Permanganate spray

A stock solution of 1% potassium permanganate was prepared and a 2% solution of sodium metaperiodate was made up as required. The two solutions were mixed in the ratio 2 vol periodate:1 vol permanganate and the resulting solution was sprayed onto the air dried chromatogram. Areas containing carbohydrate developed as brown areas on a pink background. The papers were then washed in distilled water until the background colour was removed.

(v) Toluidine Blue[75]

(vi) Ninhydrin[76]

G.M. 13 THIN LAYER CHROMATOGRAPHY

Thin layer chromatography was carried out using Whatmans cellulose CC41 (15 g.) in distilled water (40 ml) or Stahl Kieselgel G (30 g.) in distilled water (60 ml). All phases were spread on glass plates to a depth of 0.25 mm. The Kieselgel plates were activated for 45 minutes at 120°C before use.

SOLVENTS:- A, B, C, (G.M. 12) and J. (Butanol:Ethanol: Water, 3:1:1) for cellulose and solvent Y^{77} (benzene:ethanol, 8:2) for Kieselgel G.

G.M. 14 IONOPHORESIS

Ionophoresis has been carried out using a Shandon High Voltage Electrophoresis Apparatus. The sample to be analysed was placed on either Whatman No.1 or No.3 paper according to the amount of material available.

M_G VALUES

M_G values are quoted and are calculated as follows for the major analytical buffer (buffer (a)).

$$M_{GA} = \frac{\text{DISTANCE COMPONENT MIGRATES}}{\text{DISTANCE GLUCURONIC ACID MIGRATES}}$$

Galactose was used as the non-moving marker.

BUFFERS

The major analytical and preparative buffer used was pyridine/acetic acid. Pyridine (normal laboratory grade) was added to a well-stirred 0.05N solution of acetic acid (2L.) until the pH of the solution was between 6.20 and 6.50.

G.M. 15 GAS LIQUID CHROMATOGRAPHY (g.l.c.)

A INSTRUMENTATION AND COLUMNS

The instrument used for the majority of the work was a Pye Argon Chromatograph fitted with an Argon ionisation detector using dry argon as the carrier gas. The gas flow and temperature were varied according to the specific task. For certain quantitative analysis a Perkin Elmer Chromatograph and flame ionisation detector were used with nitrogen as the carrier gas.

The major columns used for analytical procedures were:-

(1) Butane-diol-succinate polyester (B.D.S.) 15%

(2) Polyphenyl ether [m-bis-(m-phenoxy-phenoxy)benzene](PPE)10%

(3) S.E. 30 3%

(4) Apiezon K (ApK) 10%

All liquid phases were coated on celite supports which were previously acid and alkali washed and coated with hexa-methyldisilazane.

B PREPARATION OF TRIMETHYL SILYL ETHERS

(i) SIL-PREPS

The material for analysis (5-10 mg.) was dried and to it was added the contents of one Sil-Prep capsule [1 ml containing trimethyl silyl chloride (1 vol), hexamethyldisilazane (3 vol) and pyridine (9 vol)] and the solution heated at 40°C for 45 minutes after which the solvent was evaporated and the residue dissolved in ether and analysed by g.l.c.

(ii) USE OF TRIMETHYL SILYL CHLORIDE AND HEXAMETHYL-DISILAZANE[78]

The material for analysis (ca. 5-10 mg.) was dried, dissolved in dry pyridine (1 ml) and to the solution was added trimethyl silyl chloride (0.1 ml) and hexamethyldisilazane (0.2 ml).

The solution was shaken for several minutes and then centrifuged. The supernatant was removed and evaporated to dryness. This residue was dissolved in ether and analysed by g.l.c.

C ACETYLATION

Acetylation was carried out by the method of Björndal, Lindberg and Svensson.[79]

D CONDITIONS

Trimethyl silyl ethers of aldoses, pentitols and hexitols were analysed on columns (3) and (4) at 155°C. The same derivatives of glycerol and threitol were analysed on the same columns at 125°C. The flow rate was constant ca. 40 cc/min. The quantity of material applied to the column was $2\text{-}10\times10^{-6}$g.

Methylated glycosides and silylated methylated glycosides were examined under the conditions used by Aspinall.[80]

Acetyl derivatives of methylated materials were analysed using columns (3) and (4) at 179°C.

G.M. 16 MICRO-PERIODATE OXIDATION OF REDUCED OLIGOMERS

The method of Belcher et al.[81] was used to determine the quantity of periodate reduced during oxidation of reduced oligomeric species, the oxidation occurring specifically at the terminal alcohol unit.

G.M. 17 REDUCTION OF ALDEHYDE GROUPS TO PRIMARY ALCOHOLS

This was carried out using 2% aqueous potassium borohydride solution unless otherwise specified.

PART ONE

SOME STUDIES ON THE HOMOGENEITY OF THE HOT WATER-SOLUBLE POLYSACCHARIDE EXTRACT FROM *CLADOPHORA RUPESTRIS*

EXPERIMENT 1. EXTRACTION OF POLYSACCHARIDE

Air dried Cladophora rupestris (800 g.) collected at Penmon Point, Anglesey, in September, 1967, was frozen in liquid nitrogen, ground to a fine powder and passed through a fine sieve (50 mesh) into n-butanol one third saturated with water (2L). After one day the solid was strained and replaced in a fresh solution of n-butanol. The solution was filtered after the same period and the residual weed placed in acetone (2L) for 1 day (x 10) and finally dried. A pale brown powder resulted.

The powder was extracted with boiling water (800 ml) for 2 hours under an atmosphere of nitrogen and the various extracts separated from the residue while hot by straining through muslin. The procedure was repeated (x 5) until the extract contained negligible carbohydrate. The extracts were combined, cooled, and centrifuged to remove extraneous cell debris (2500 r.p.m.) for 15 minutes.

The extract was treated with human salivary amylese (50 ml) with the solution at a concentration of 0.1% sodium chloride. After 24 hours when a negative test for starch was obtained the solution was brought to a concentration of 4% with respect to trichloroacetic acid and left for 12 hours

at 0°C. The precipitated protein was centrifuged, the supernatant liquid neutralized with 5N-sodium hydroxide and dialysed extensively against tap water and then distilled water. The non-dialysable component was concentrated to small bulk, the polysaccharide precipitated with alcohol (1 vol excess), centrifuged, dissolved in water and freeze-dried (40 g.).

It had:-

Carbohydrate content = 50%

Sulphate content = 21%

Nitrogen content = 2.07% (x 6.25 for protein)

$[\alpha]_D^{20^\circ}$ = +56°

EXPERIMENT 2. EXAMINATION OF EXTRACT BY GEL COLUMN CHROMATOGRAPHY

Polysaccharide (ca. 5-10 mg) was dissolved in 1.5M-sodium chloride (1 ml) and applied to a Sagavac column (see EXPT. 13) (90 cm x 1 cm) and eluted with 1.0M-sodium chloride; 2 ml fractions were collected. The fractions were analysed for protein content (at 280 mμ) and carbohydrate content (G.M. 5). A graph showing the results of these analyses is given (Figure 2).

The eluates in tubes 25 to 55 were collected in three separate fractions 25-35(A), 36-42(B), and 43-55(C). All three were extensively dialysed, concentrated to small bulk and freeze-dried. They were hydrolysed (G.M. 8 (a)) and analysed by paper chromatography (solvents A and B) which revealed the presence of galactose, arabinose and xylose in all fractions. A visual estimation of the relative proportions of sugars is given below in TABLE IV.

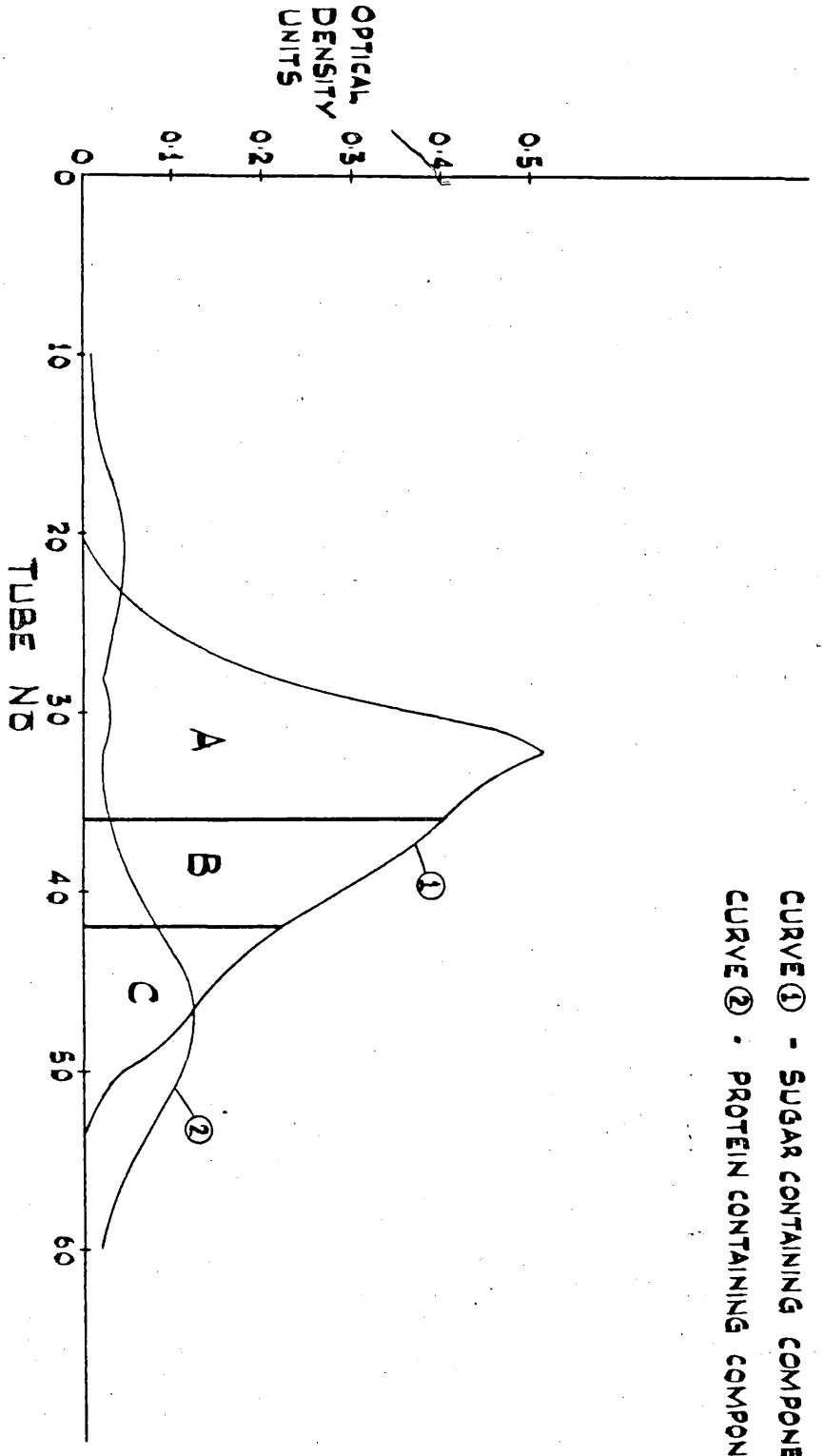
	GALACTOSE	ARABINOSE	XYLOSE	RHAMNOSE
A	+++	+++	+	TRACE
B	+++	+++	+	"
C	++	+	±	"

TABLE IV

EXPERIMENT 3. EXAMINATION OF EXTRACT BY GEL ELECTROPHORESIS

Electrophoresis on polyacrylamide columns was carried out according to the method of Stewart, Lyndon and Barber[82] with a Shandon apparatus. Reservoir buffer (tris-glycine, pH 8.3) was prepared as described by Davis.[83] Polysaccharide (0.025 mg. x 2) in 4% sucrose solution was applied to the polyacrylamide columns (x 2) and electrophoresis of both was carried out for one hour at 250 v and 3mA. One tube was

POLYSACCHARIDE FRACTIONATION ON SAGAVAC



CURVE ① - SUGAR CONTAINING COMPONENT

CURVE ② - PROTEIN CONTAINING COMPONENT

FIGURE 2

stained with methylene blue and the other with toluidine blue by leaving the extruded columns in the reagents for one hour and then washing to remove excess stain. Single coincidental bands (3 mm wide) were obtained for protein and carbohydrate.

EXPERIMENT 4. EXAMINATION OF EXTRACT BY ULTRA-FILTRATION

A sample of starch-containing polysaccharide (100 mg) was taken up in water (20 ml) and placed inside a Sartorius pressure filter apparatus containing a cellulose filter of known pore diameter (250mμ). A supply of nitrogen was fixed to the unit and the polysaccharide solution filtered through at a pressure of 70-100 lb/sq.in. The filtrate was collected as was the sediment retained on the filter (K), the sediment was freeze-dried and hydrolysed (G.M. 8 (a).

The filtrate from the procedure was filtered in the same manner, sequentially, through cellulose filters with average pore diameters of 150 , 20-30 , and 5mμ. All of the sediments and the final filtrate were freeze-dried and hydrolysed (G.M. 8 (a). TABLE V gives the results of paper chromatographic analysis of these hydrolysates.

FILTER	% WEIGHT RETURNED	GALACTOSE	ARABINOSE	XYLOSE	RHAMNOSE
250 mμ	80%	+++	+++	+	TRACE
150 "	10%	+++	+++	+	"
20-30 "	8%	++	+++	+	"
5 "	2%	GLUCOSE			
5 " FILTRATE	0%	NO CARBOHYDRATE			

TABLE V

PART TWO

SMITH'S DEGRADATION STUDIES ON THE HOT WATER-SOLUBLE POLYSACCHARIDE EXTRACT FROM *CLADOPHORA RUPESTRIS*

EXPERIMENT 5. FIRST SMITH'S DEGRADATION

Polysaccharide, 6.5 g., (carbohydrate content - 50%) was taken up in water (1 litre) and to this solution aqueous 0.03M sodium metaperiodate (1 litre) was added. A blank (1 ml) was withdrawn immediately and aliquots (1 ml) of the solution sampled at regular intervals and examined for reduction of periodate (G.M. 4). After 27 hours the reaction was stopped by addition of ethane 1-2 diol (20 ml). The extent of reduction is shown graphically (Figure 3). The primary oxidation determined by extrapolation to zero time was 0.36M/anhydro hexose unit.

A small aliquot (10 ml) of the solution was treated with 0.05M boric acid and 2% potassium borohydride at 0°C to ascertain whether side reactions occurred. It was found that no coloured compounds were produced and so the rest of the solution was treated in exactly the same manner and left at 0°C for 48 hours. Total reduction had occurred since an aliquot after neutralisation with analar glacial acetic acid gave a negative Fehling's test. The whole solution was neutralised and dialysed extensively against distilled water. The polyalcohol (Polyalcohol I) was concentrated to small bulk and precipitated with alcohol (1 vol). The precipitate, separated by centrifugation, was dissolved in water and freeze-dried (6.53 gms., carbohydrate content = 38%).

The polyalcohol was taken up in water (500 ml) and IR 120(H^+) resin added until the pH reached 1.5. The acid solution was well stirred for 24 hours. At the end of this period the solution was neutralised with ammonia solution after the resin had been removed by filtration. The solution was concentrated to small bulk and the polymeric material precipitated with alcohol (1 vol). The precipitated degraded polymer (degraded polymer I) was separated by centrifugation and this solid was dissolved in water and freeze-dried (4.53 g. carbohydrate content = 45%). The alcoholic supernatant separated from centrifugation was evaporated to small bulk and freeze-dried (1.27 g. carbohydrate content = 19.1%).

EXPERIMENT 6. SECOND SMITH'S DEGRADATION

Degraded polymer I (4.53 g) was periodate oxidised as in the previous experiment and the extent of oxidation is shown graphically (Figure 3). The reduction of periodate from primary oxidation was 0.24 moles/anhydrohexose unit.

The material was reduced with potassium borohydride as before and the polyalcohol purified by dialysis and precipitation with alcohol. The polyalcohol (Polyalcohol II) was freeze-dried (4.30 g, carbohydrate content = 35%).

Polyalcohol II was mildly hydrolysed using the autohydrolysis technique exactly as before and the fragments (1.7 g.carbohydrate content = 40.8%) and degraded polymer II

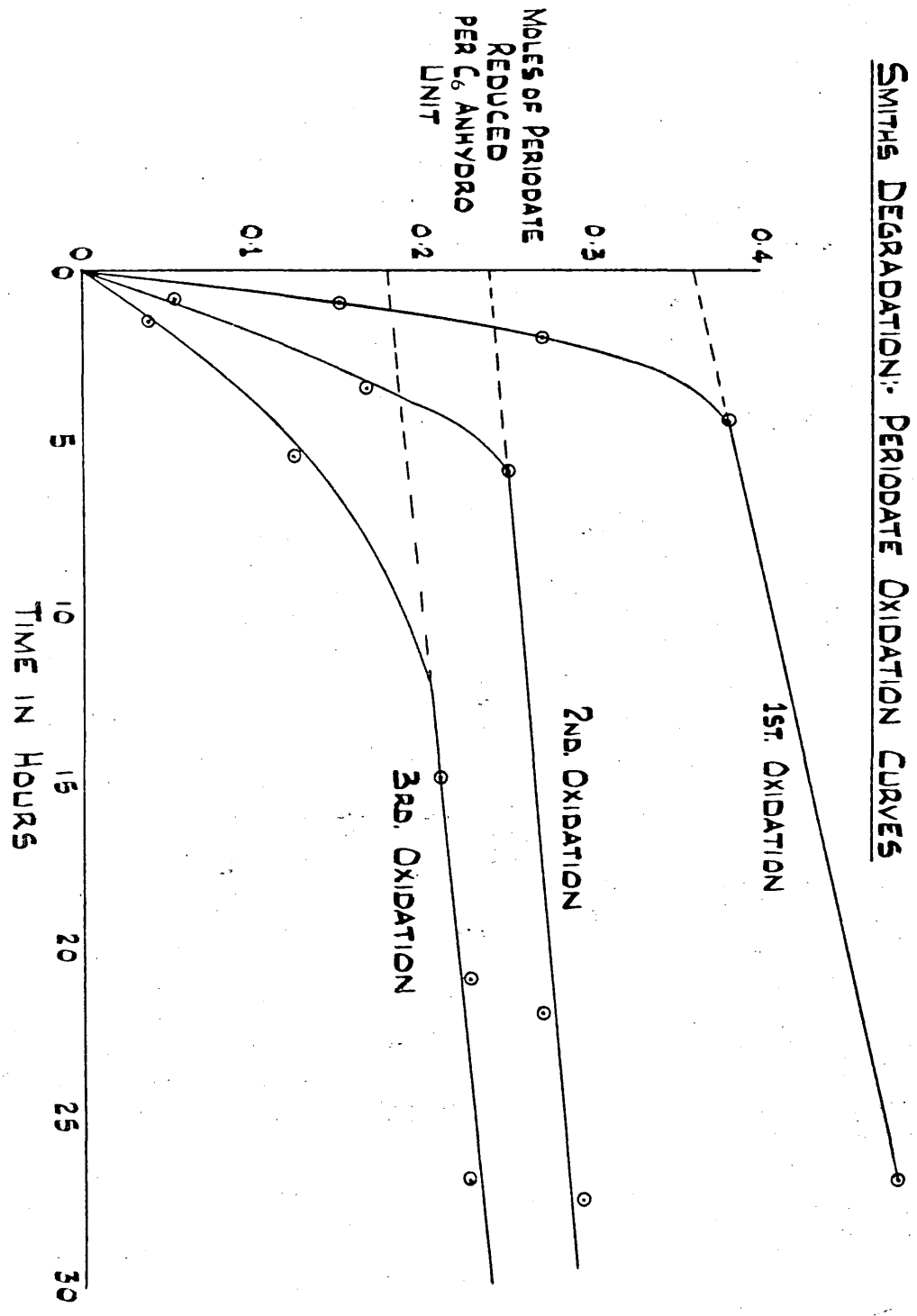

SMITHS DEGRADATION: PERIODATE OXIDATION CURVES
MOLES OF PERIODATE REDUCED PER C6 ANHYDRO UNIT
0
0.1
0.2
0.3
0.4
0
5
10
15
20
25
30
TIME IN HOURS
1ST. OXIDATION
2ND. OXIDATION
3RD. OXIDATION

FIGURE 3

(1.7 g. carbohydrate content = 38%) separated and freeze-dried as before).

EXPERIMENT 7. THIRD SMITH'S DEGRADATION

The degraded polymer II (1.70 g.) was periodate oxidised as in the previous experiments and the extent of oxidation is shown graphically (Figure 3). The reduction of periodate from primary oxidation was 0.18M/anhydrohexose unit.

The material was reduced and purified as before and polyalcohol (Polyalcohol III) was recovered after freeze-drying (1.63 g, carbohydrate content = 34%).

Polyalcohol III was subjected to mild hydrolysis as before with the exception that a small volume of sulphuric acid had to be added to lower the pH to 1.5. Degraded polymer III (1.13 g., carbohydrate content = 28.5%) and the fragments (0.85 g, carbohydrate content = 17.1%) were recovered.

EXPERIMENT 8. SMALL SCALE COMPLETE HYDROLYSIS OF POLYALCOHOL I

Polyalcohol I (ca. 2mg.) was hydrolysed (G.M. 8 (a)) and the hydrolysate examined by g.l.c. as the trimethylsilyl ethers and by paper chromatography (solvent A and B) stain (i) and (ii). Components were revealed with the characteristic mobility and colour of glycerol, a tetritol, threitol, arabinose, galactose, and a trace of rhamnose.

EXPERIMENT 9. PREPARATION OF L-THREITOL

n-Butyl-L-tartrate (5g) was dissolved in redistilled dry tetrahydrofuran (25 ml). This solution was added slowly with stirring to a suspension of fresh lithium aluminium hydride (3g) in a mixed solvent of tetrahydrofuran and ether (75 ml: 25 ml). The mixture was heated under reflux for 4 hours and then cooled and poured into water (150 ml).

Glacial acetic acid was added and the solution was evaporated to dryness under reduced pressure. Acetic anhydride (100 ml) was added and the slurry refluxed for one hour. The solution was evaporated to dryness and then the solid residue was shaken with water acidified with 0.1N-hydrochloric acid. This liquid was extracted with chloroform (3 x 200 ml). The combined chloroform extracts were washed with water (2 x 400 ml) and dried over solid anhydrous magnesium sulphate.

The chloroform was evaporated to dryness and the resulting solid was deacetylated by refluxing at 100°C for one hour with a mixture of N-sodium hydroxide (75 ml) and ethanol (50 ml). The resulting liquid was deionised with IR 120(H^+) resin and the neutral solution evaporated to a syrup which slowly crystallised. The crystalline solid was recrystallised from ethanol/ethylacetate (1:1 v/v).

YIELD = 0.64 g.

M.Pt. = 84-88° (M.Pt. THREITOL = 88°).[85]

Preparation of 1,3-monobenzylidene-L-threitol

L-Threitol (100 mg.) was shaken for 1 hour with a solution of 50% sulphuric acid (1 ml) and benzaldehyde (1 ml). The reaction mixture was extracted twice with chloroform and evaporated. The residue was dissolved in methanol (0.5 ml) and an equal volume of water added. Small needle shaped crystals separated immediately. They were recrystallised from toluene.

M.Pt. = 132-133° (1,3-monobenzylidene-L-threitol M.Pt. = 133°C)[86]

EXPERIMENT 10. PREPARATION OF D-THREITOL FROM POLYALCOHOL I

Polyalcohol I (500mg.) was hydrolysed with 90% formic acid (10 ml, G.M. 8 (a)). The syrupy hydrolysate was applied to two No.17 papers and eluted with solvent A for 24 hours. The position of the tetritol was determined by cutting side and centre strips and spraying with reagent (i). It was eluted with distilled water and the eluate evaporated to small bulk and treated with bromine (5 drops) to oxidise contaminating sugars.

To ensure satisfactory separation of the threitol from the acid contaminants a trial separation of a mixture of synthetic galacturonic acid and threitol (10 mg. of each) was carried out. The synthetic mixture in water (1 ml) was

applied to a column (10 x 1 cm) of IRA 400 (OH^-) resin and the column eluted with distilled water. The aqueous eluate was evaporated and crystals of L-threitol spontaneously crystallised (M.Pt. 79-81°).

The solution containing threitol and acids from the above bromine oxidation was aerated to remove excess bromine. The strongly acidic solution was neutralised with silver carbonate and filtered. The filtrate was treated with hydrogen sulphide and the precipitated silver sulphide centrifuged off. The supernatant was retained and evaporated to a syrup (X).

The syrup (X) was taken up in water (1 ml) and applied to a freshly prepared IRA 400 (OH^-) column (10 x 1 cm) which was then eluted with distilled water and the eluate evaporated to dryness. The syrup slowly crystallised (M.Pt. 82-88°C) (cf. L or D Threitol M.Pt. 86-88°C).

The crystalline solid (ca. 10 mg.) was converted to the 1,3-monobenzylidene derivative as before. (M. Pt. 130°C), (cf. 1,3-monobenzylidene-L-threitol M.Pt. = 132-133°C). The derivatives were analysed by g.l.c.(Column (4)) as the trimethylsilyl ether and the synthetic and unknown materials were found to have identical retention times as follows:

	R_{TH}
2,4 disilyl, 1,3-monobenzylidene-L-threitol	2.30
Unknown derivative	2.30

R_{TH} = Retention time relative to that of threitol

EXPERIMENT 11. PREPARATION OF CALIBRATION CURVES FOR g.l.c. ANALYSIS OF SUGAR RESIDUES AND SUGAR FRAGMENTS

The sugar residues galactose, arabinose, xylose and rhamnose were known to exist in the native polysaccharide[56] and the sugar fragments glycerol and threitol were detected in earlier experiments. Standard samples of all these components were converted to the trimethylsilyl derivative and used to prepare calibration curves for quantitative g.l.c. using column (3).

The standards (ca. 3-5 mg.) were weighed accurately, the glycerol being well dried beforehand by evaporation from dry methanol and dry benzene solutions. The trimethylsilyl ethers were prepared using Sil-Preps (G.M. 15 B (i)) and the derivatives dissolved in accurately measured volumes of cooled dry ether (2-5 ml) and the solutions stored at low temperature (-20°C). Accurately known aliquots (0.1-1 μl) were injected at the head of the column and the peak areas and peak heights of the resulting peaks examined. Peak area was calculated from the normal triangulation procedure:-

Area = peak height x peak width at half peak height

Peak area was plotted against weight of standard and typical graphs are illustrated (Figure 4).

-g.l.c. CALIBRATION CURVES

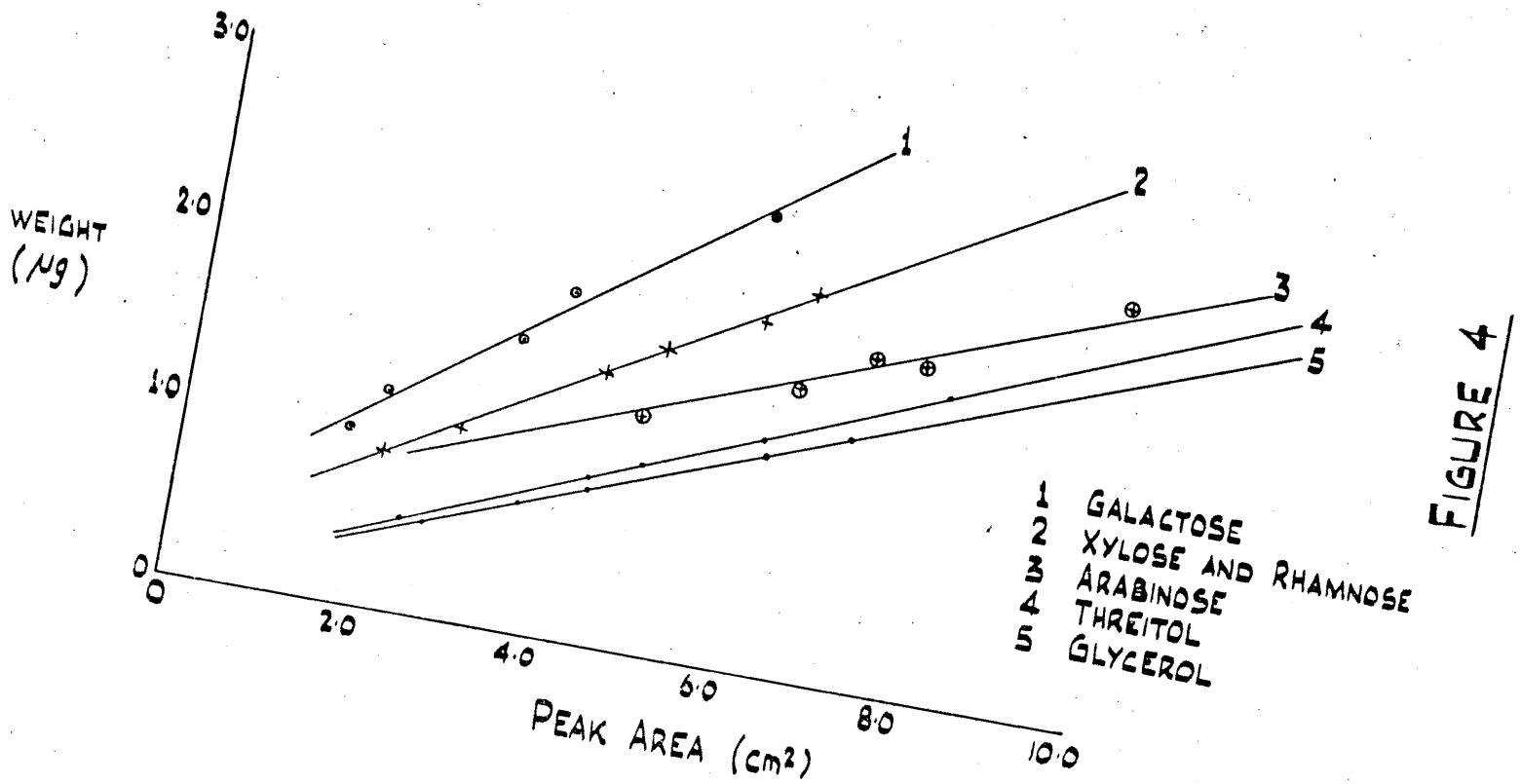


FIGURE 4

The retention times for the standards relative to that of threitol are shown below:-

	R_{TH}
GALACTOSE	4.02, 5.33
ARABINOSE	1.64, 1.95
XYLOSE	2.77, 3.77
GLYCEROL	0.41
THREITOL	1.00

EXPERIMENT 12. ANALYSIS OF SOME POLYMERIC SPECIES FROM THREE SMITH'S DEGRADATIONS BY *g.l.c.*

Polysaccharide, polyalcohol I, degraded polymer I, etc. (5 mg. each) were hydrolysed individually (G.M. 8 (a)) and the hydrolysates dried, converted to the trimethylsilyl ether (G.M. 15 B(ii)) and examined by g.l.c. (column (3)). The chromatogram from the recorder was analysed and the peaks assigned to those of previously examined standards. The areas under the peaks corresponding to each component were measured as for previous experiment and the weight equivalent estimated from the relevant calibration curve. The results are shown in TABLE VI.

		GALACTOSE	ARABINOSE	XYLOSE	THREITOL	GLYCEROL
POLYSACCHARIDE	R.P.	1.00	0.83	0.36	-	-
	M.P.	1.00	1.00	0.44	-	-
POLYALCOHOL I	R.P.	1.00	1.00	-	0.09	0.23
	M.P.	1.00	1.20	-	0.12	0.40
DEGRADED POLYMER I	R.P.	1.00	1.57	-	-	-
	M.P.	1.00	1.90	-	-	-
POLYALCOHOL II	R.P.	1.00	2.90	-	0.11	0.74
	M.P.	1.00	3.50	-	0.15	1.30
DEGRADED POLYMER II	R.P.	1.00	2.32	-	-	-
	M.P.	1.00	2.80	-	-	-
POLYALCOHOL III	R.P.	1.00	1.96	-	0.21	0.54
	M.P.	1.00	2.36	-	0.28	0.94
DEGRADED POLYMER III	R.P.	1.00	2.27	-	-	-
	M.P.	1.00	2.74	-	-	-

R.P. = RELATIVE PROPORTIONS

M.P. = RELATIVE MOLAR PROPORTIONS

TABLE VI

EXPERIMENT 13. MOLECULAR WEIGHT ANALYSIS OF POLYMERIC MATERIALS FROM SMITH'S DEGRADATION BY SAGAVAC COLUMN CHROMATOGRAPHY

A column (90 cm x 1.5 cm, made by Wrights) of Sagavac 6F (made by Seravac Ltd.) was prepared. Preswollen Sagavac 6F was placed in 1M-sodium chloride solution containing sodium azide (0.02%) and was de-aerated for 30 minutes after which it was packed in the column by gravity. The completed column was eluted with 1M-sodium chloride until the level of the Sagavac at the top of the column remained stationary (ca. 4 days). Both ends of the column were fitted with stainless steel mesh (80μ pore diameter) to prevent blockage by the fine beads.

The column was calibrated to determine the void volume and exclusion volume by passing down a suspension of lyophilised E. coli cells (2 mg) in 1.5M-sodium chloride (1 ml). This mixture contained the cellular fraction which was eluted at the void volume, and the low molecular weight proteins, which were eluted at the exclusion volume. Aliquots (2 ml) of the eluate were collected over 12 hours and each of these fractions was analysed at 280 mμ on an S.P.500. A graph was drawn of intensity measurement against aliquot number (Figure 5).

GEL COLUMN CHROMATOGRAPHY OF SOME PRODUCTS FROM THE SMITH DEGRADATION STUDIES.

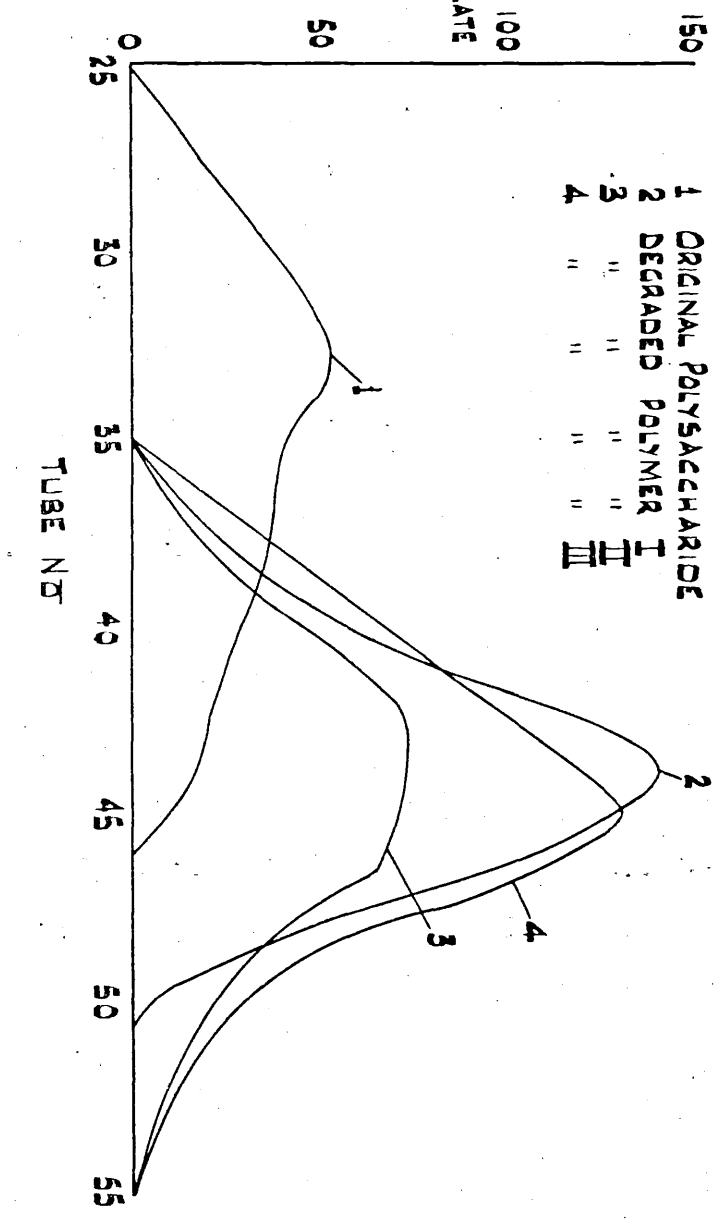


FIGURE 5

Samples of polysaccharide, degraded polymer I, degraded polymer II, and degraded polymer III (ca. 2 mg each) were dissolved in 1.5M-sodium chloride and applied to the head of the column. The column was eluted with 1M-sodium chloride and aliquots (2 ml) of eluate collected. Every fifth tube was examined for carbohydrate (G.M. 5) and once detected the aliquots to either side were examined. The intensity of the colouration produced was measured at 485 mμ using an S.P.500 spectrophotometer and these values were plotted against the relevant aliquot number (Figure 5).

From the position of the maxima of the peaks for each sample an approximate value of molecular weight was calculated and was expressed in terms of percentage of the original molecular weight. The results are shown in TABLE VII.

POLYSACCHARIDE	% OF ORIGINAL MOLECULAR WEIGHT
INITIAL POLYMER	100%
DEGRADED " I	5.1%
" " II	4.7% (Average of Plateau)
" " III	4.1%

TABLE VII

EXPERIMENT 14. ANALYSIS OF FRAGMENTS FROM DEGRADATION I, II AND III

(a) g.l.c. ANALYSIS OF FULLY HYDROLYSED FRAGMENTS I, II AND III

The fragments (ca. 3 mg) were hydrolysed (G.M. 8 (a)) and the hydrolysates dried, converted to the trimethylsilyl ethers (G.M. 15 B(ii)) and examined by g.l.c. (column 3).

The chromatograph from the pen recorder was analysed to assign the peaks by reference to previously run standards. The areas under the relevant peaks were measured and converted to weights of components by reference to previously prepared calibration curves. The results are shown in TABLE VIII.

FRAGMENTS		GALACTOSE	:	ARABINOSE
FRAGMENTS I	RELATIVE MOLAR PROPORTIONS	1	:	0.81
	RELATIVE PROPORTIONS	1	:	0.66
FRAGMENTS II	RELATIVE MOLAR PROPORTIONS	1	:	3.50
	RELATIVE PROPORTIONS	1	:	3.00
FRAGMENTS III	RELATIVE MOLAR PROPORTIONS	1	:	1.48
	RELATIVE PROPORTIONS	1	:	1.23

TABLE VIII

(b) SEPARATION INTO COMPONENTS

(i) Fragments (I)

Paper chromatography of this material in solvents A and B failed to give satisfactory resolution of the mixture on which to base preparative paper chromatographic separation. The solid (1.27g) was separated by preparative ionophoresis into acid and neutral components in buffer (i) (G.M. 14) on 12 No.3 papers (12.6 cm. width). The two fractions were eluted separately, reduced to small bulk and freeze-dried.

Paper chromatographic analysis of both components in solvent A indicated that preparative separation was feasible. The neutral and acidic fragments were applied separately to No.17 papers and developed for 30 hours (solvent A). The components were detected and isolated in the usual way (G.M. 12) as freeze-dried materials. Eight syrupy fractions (six neutral and two acidic) were separated and analysed.

(ii) Fragments II

The fragments (1.7 g) were separated directly using preparative paper chromatography (solvent A, Whatmans No.17 papers, 30 hours). The components were located and isolated as before. Six freeze-dried materials were isolated in this manner.

(iii) Fragments III

The fragments (0.85 g.) were separated by preparative paper chromatography as before. The components were located and isolated in a manner analogous to that used previously. Four syrupy fractions were isolated.

(c) ANALYSIS OF SEPARATED FRAGMENTS

(i) Fragments I

Fraction IA

Glycerol, R_{GAL} 1.95 (solvent A); 2.3 (solvent B) spray (i), identical with standard glycerol. This was confirmed by g.l.c. (column 3 and 4, G.M.15) of the T.M.S. derivative.

Fraction IB

Threitol, R_{GAL} 1.66 (solvent A); 1.80 (solvent B) spray (i), identical with standard threitol, confirmed by g.l.c. of the T.M.S. derivative.

Fraction II

R_{GAL} 1.42 (solvent A) spray reagent (i), gave on hydrolysis arabinose and glycerol (solvents A and B) spray (i).

Fraction III

R_{GAL} 1.20 (solvent A) spray (i), gave on hydrolysis arabinose and threitol. Confirmed by g.l.c. as the T.M.S. derivative in a molar ratio of 1:1.

Neutral fractions IV and V and acidic fractions I and II gave varying quantities of glycerol, threitol, galactose, and arabinose (solvents A and B, spray (i)). They had the respective mobilities and molar proportions shown in TABLE IX.

FRACTION	R_{GAL} (Solvent A)	M_{GA} (Buffer(i))	MOLAR PROPORTIONS BY g.l.c. OF THE T.M.S. DERIVATIVES (COLUMN 3)			
			GALACTOSE	ARABINOSE	THREITOL	GLYCEROL
NEUTRAL IV	0.97, 1.22	-	1.0	0.42	0.47	1.0
NEUTRAL V	0.65	-	1.0	1.05	1.0	0.77
ACIDIC I	0.47	1.0	1	0.7	0.8	1.5
ACIDIC II	0.2-0.4	1.33(MAJOR)	1	1.5	0.3	1.7

TABLE IX

(ii) Fragments II

Fraction I

Threitol, R_{GAL} 1.66 (solvent A); 1.80 (solvent B) spray (i); identical with standard threitol and confirmed by g.l.c. of the T.M.S. derivative.

Fraction II

R_{GAL} 1.42(solvent A) gave on hydrolysis arabinose and glycerol (solvents A and B) spray (i).

Fractions III-V on hydrolysis gave varying proportions of galactose, arabinose, threitol, and glycerol and fraction VI gave ~~only~~ galactose, arabinose and rhamnose (trace) (solvents A and B, spray (i)). They had the properties tabulated below in TABLE X.

FRACTION	R_{GAL} (Solvent A)	M_{GA} (Buffer(i))	MOLAR PROPORTIONS BY g.l.c. AS T.M.S. DERIVATIVE (COLUMN 3)			
			GALACTOSE	ARABINOSE	THREITOL	GLYCEROL
III	0.58	1.40(MAJOR)	1	: 4.2	1	: 4.5
IV	0.52	1.0, 1.4 (TRACE)	1	: 2.3	1	: 6.5
V	0.2 - 0.3	1.0, 1.2, 1.3	1	: 3.5	2.0	: 10.3
VI	0 - 0.1	0.1 - 1.0	1	: 3 (visual estimation, paper chromatography). This fraction represented 75% of total carbohydrate in the fragments.	TRACE	: TRACE

TABLE X

(iii) Fragments III

Fraction I

Threitol, R_{GAL} 1.66 (solvent A); R_{GAL} 1.80 (solvent B) spray (i), identical with standard threitol and confirmed by g.l.c. of the T.M.S. derivative.

Fraction II

R_{GAL} 1.42 (solvent B) gave on hydrolysis arabinose and glycerol only (solvents A and B) spray (i).

Fractions III and IV on hydrolysis gave varying proportions of galactose, arbinose, threitol, and glycerol. They had the respective mobilities and molar proportions listed below in TABLE XI.

FRACTION	R_{GAL} (Solvent A)	M_{GA} (Buffer (i))	MOLAR PROPORTIONS BY g.l.c. AS T.M.S. DERIVATIVE (COLUMN 3)			
			GALACTOSE	ARABINOSE	THREITOL	GLYCEROL
III	0.58	1.00	1.00	1.90	0.93	2.00
IV	0.45	2.00	1.00	1.00	1.00	0.90

TABLE XI

EXPERIMENT 15. ANALYSIS OF DEGRADED POLYMER FROM THE THIRD SMITH'S DEGRADATION

The final product from the sequential Smith's degradation (degraded polymer III) had, SO_4^{2-} 30.5% (G.M. 7) and was only partially soluble in water. The water soluble and insoluble components were separated by centrifugation. The solid was dispersed in water and freeze-dried (400 mg.). The water soluble component, after extensive dialysis, was evaporated

to small bulk, precipitated with alcohol (1 vol), dissolved in water and freeze-dried (720 mg., 36% SO_4, 40% carbohydrate).

This component was subjected to examination by D.E.A.E. paper chromatography. Whatman DE82 cellulose paper (15 cm x 1.5 cm) was eluted with 1M-potassium chloride by the descending elution technique. The thoroughly wetted paper was blotted, a sample of polymer spotted on, and the paper eluted for 30 mins. After drying it was developed with spray reagent (v). Only a part (ca. 40%) was eluted by the buffer. The procedure was repeated on a fresh chromatogram eluting in this instance with 3M-potassium chloride. A small portion of the polymer was retained on the baseline, and even attempted separation using an alkaline eluant (0.5M-sodium hydroxide) failed to move this component.

EXPERIMENT 16. ANALYSIS OF DEGRADED POLYMER III BY DIETHYL-AMINO-ETHYL (D.E.A.E.)-CELLULOSE COLUMN CHROMATOGRAPHY

Diethyl-amino-ethyl (D.E.A.E.)-cellulose (100 g.) was washed with constant agitation in 0.5M-hydrochloric acid (500 ml) under vacuo. After 20 minutes the solid was allowed to settle and the supernatant containing fines was decanted. The residue was then filtered at the pump and washed with water to neutrality. The acid washed cellulose was similarly

treated with 0.5M-sodium hydroxide and washed to neutrality with water. The residue was washed a further three times with acid and alkali as before except that the final aqueous washing was omitted.

The alkaline, de-aerated cellulose was packed under gravity in a column (60 cm x 5 cm) which was filled at the bottom with a layer of glass wool (5 cm) and a layer of acid and alkali washed sand (7 cm). The packed column was washed with water until neutral and then eluted with 0.5M-sodium chloride (2L). Finally it was washed with water (2L) until a test for chloride ion was negative.

The degraded polymer (700 mg), in water (10 ml), was applied to the top of the column, and allowed to equilibrate for 2 hours. It was then eluted with water, 25 ml fractions were collected and every third fraction was analysed for carbohydrate (G.M. 5). No carbohydrate was detected in the aqueous eluate.

The column was sequentially eluted with 0.5M-, 1.0M-, 1.5M-, 3.0M-potassium chloride, and 0.5M-sodium hydroxide. All the eluents were examined for carbohydrate (see Figure 6)(p88).

The following fractions were obtained after dialysis and freeze-drying, and the results shown in TABLE XII.

		WEIGHT	SULPHATE	CARBOHYDRATE
FRACTION I	- 0.5-M-KCl	242 mg.	28.5%	50%
" II	1.0 "	68 mg.	32.1%	42%
" III	1.5 "	10 mg.	-	30%
" IV	3.0 "	-	-	-
" V	0.5-M NaOH	-	-	-

TABLE XII

Paper chromatography of hydrolysates (Solvents A and B, spray (i) and (ii)) revealed the presence of galactose, arabinose and rhamnose (trace) in all fractions, estimated visually to be as shown in TABLE XIII.

	0.5 M-KCl	1.0M-KCl	1.5M-KCl
GALACTOSE	+	+	+
ARABINOSE	++	++	+
RHAMNOSE	TRACE	TRACE	TRACE

TABLE XIII

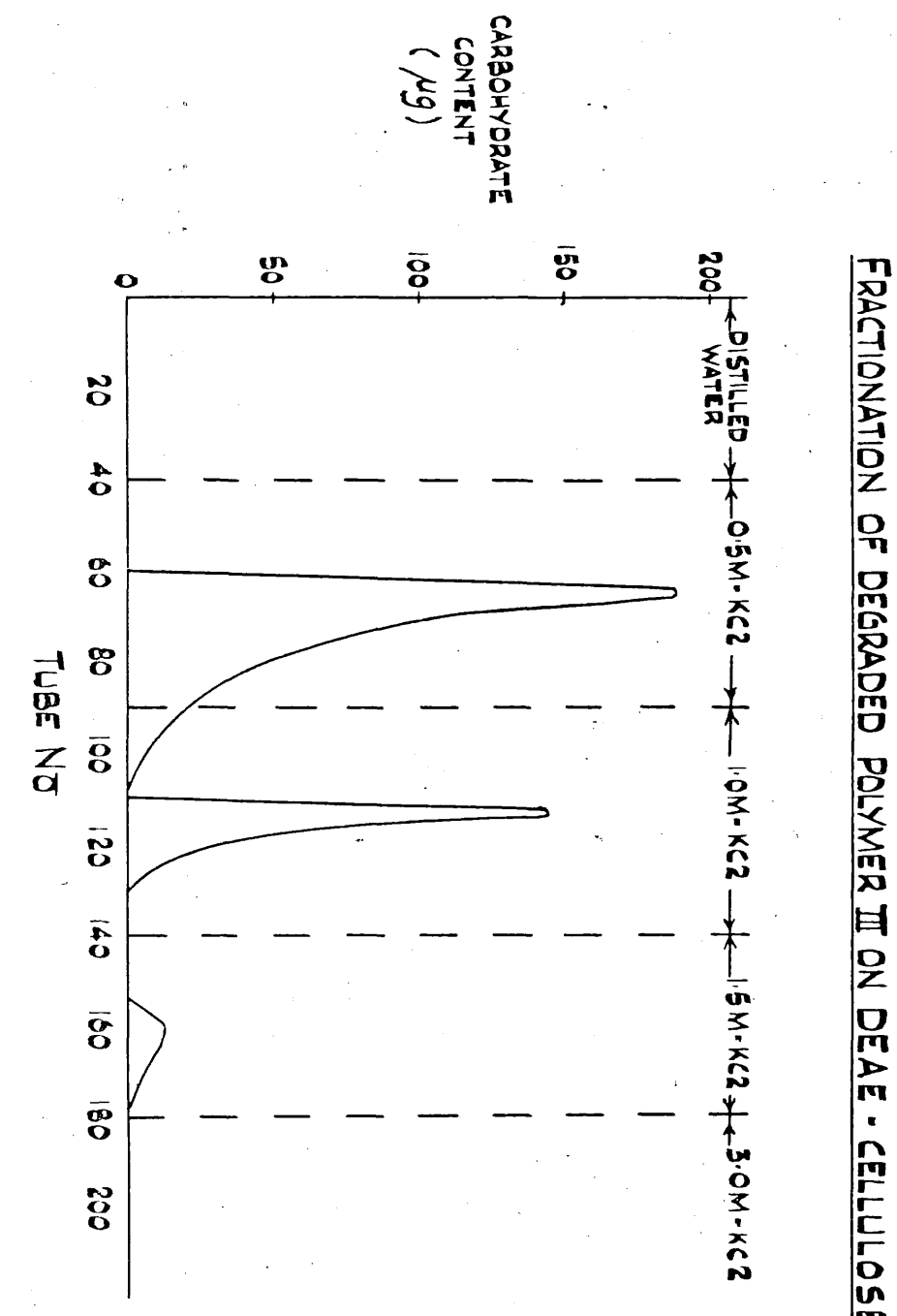

FRACTIONATION OF DEGRADED POLYMER III ON DEAE - CELLULOSE
CARBOHYDRATE CONTENT (µg)
0
50
100
150
200
DISTILLED WATER
0·5M-KCl
1·0M-KCl
1·5M-KCl
3·0M-KCl
20
40
60
80
100
120
140
160
180
200
TUBE NO

FIGURE 6

PART THREE

PARTIAL ACID FRAGMENTATION STUDIES ON THE HOT WATER-SOLUBLE EXTRACT FROM *CLADOPHORA RUPESTRIS*

EXPERIMENT 17. PARTIAL AUTOHYDROLYSIS A

Polysaccharide (22.8 g., carbohydrate content 40%) dissolved in water (1000 ml) was converted into its free acid form by passage through a column (3 cm x 50 cm) of Amberlite IR 120 (H^+) resin. The solution recovered from this process was placed inside a dialysis sac, which was then suspended in a vessel containing distilled water maintained at 100°C. The well stirred dialysate was removed after two hours and replaced with fresh distilled water. This was repeated twice.

The combined dialysates after neutralisation (.880 ammonia solution) were concentrated to a thick syrup (7g., carbohydrate content ca. 20%). The non-dialysable fraction was separated into an insoluble component (5.2 g., carbohydrate content 5-10%, N_2 6-7%), which had precipitated in the sac during the reaction, and a soluble component (9.8 g., carbohydrate content 55%). Both were isolated after neutralisation (.880 ammonia solution). A hydrolysate of the soluble component contained arabinose (major sugar), galactose, and xylose (equal), and rhamnose (trace) (paper chromatography).

The syrupy dialysate (7 g.) carbohydrate content 20% in water (10 ml) was separated from acidic contaminants on an Amberlite IRA 400 ($acetate^-$) resin column (3 cm x 50 cm).

The neutral material, eluted with water (2L), on evaporation gave a syrup (900 mg; carbohydrate content 33%). This was fractionated on a charcoal-Celite column (1:1, acid washed). The column was eluted sequentially with water (14L), 5% (5.2L) 10% (7.3L) and 15% (2.5L) of aqueous ethanol and the eluant collected in 25 ml fractions. Every tenth tube was analysed for carbohydrate content and by paper chromatography. The aqueous solution contained the four constituent monosaccharides with traces of oligosaccharides in the later fractions. Of the remaining eluants only the 5% aqueous ethanol contained carbohydrate. This was concentrated to a syrup (60 mg.) and separated into its components on Whatman No.1 paper (solvent E).

EXPERIMENT 18. ANALYSIS OF NEUTRAL OLIGOMERS FROM PARTIAL AUTOHYDROLYSIS A

COMPONENT I

A syrup (6.8 mg.), R_{GAL} 1.05 (solvent A); 0.68 (solvent B); 0.89 (solvent E), $[\alpha]_D = -39^\circ$, (c, 0.68) was homogeneous in all solvents. It had a DP of 2 and gave a pink brown colour with spray reagent (ii). Paper chromatography (solvents A and B; spray reagents (ii)) of a hydrolysate (G.M. 8(a)) revealed the presence of galactose and xylose.

Analysis of the reduced and hydrolysed fragment by g.l.c. as the TMS (G.M. 15 B (i)) on columns (2,3 and 4) were poor but xylitol was definitely identified. An aliquot (ca.2 mg.) was methylated, converted into the methyl glycoside, and examined by g.l.c. (columns 1 and 2), peaks consistent with the retention times of methyl 2,3,4-tri-O-methylxyloside, methyl 2,3-di-O-methylxyloside, and methyl 2,3,5,6-tetra-O-methylgalactoside were obtained.

COMPONENT II

A syrup (9.1 mg.), R_{GAL} 0.96 (solvent A), 0.68 (solvent B) and 0.50 (solvent E), $[\alpha]_D = -18^\circ$ (c, 0.9), was homogeneous in all solvents. It had a DP of 2 and gave a pink brown colour with spray reagent (ii). Paper chromatography (solvents A and B; spray reagents (i) and (ii)) of a hydrolysate (G.M. 8(a)) revealed the presence of galactose and xylose. Analysis of the hydrolysed reduced oligomer by g.l.c. as the TMS ether (G.M. 15 B(ii)) (columns 2,3, and 4) indicated the presence of galactitol, galactose, and xylose. An aliquot (ca. 2 mg.) was methylated, converted to the methyl glycoside, and examined by g.l.c. (columns 1 and 2). Peaks with retention times identical with those of standard methyl 2,4,6-tri-O-methylgalactoside, methyl 2,3,5,6-tetra-O-methyl-galactoside, and methyl 2,3,4-tri-O-methyl xyloside were obtained.

COMPONENT III

A syrup (7.2 mg.), R_{GAL} 0.87 (solvent A), 0.40 (solvent B) 0.35 (solvent E), was homogeneous in all solvents and identical with 1,6 galactobiose and had[55] $[\alpha]_D$ = + 11° (c, 0.7) (cf. +32° for 1,6-galactobiose) and gave a brown coloured spot with spray reagent (ii). Paper chromatography (solvents A and B; spray reagents (i) and (ii) of a hydrolysate (G.M. 8(a)) revealed the presence of galactose only. An aliquot (ca. 2 mg.) was methylated and converted to the methyl glycosides by methanolysis. Analysis of the mixture by g.l.c. (columns 1 and 2) gave peaks with retention times consistent with methyl 2,3,4-tri-O-methylgalactoside, and methyl 2,3,4,6-tetra-O-methyl galactoside.

EXPERIMENT 19. PARTIAL AUTOHYDROLYSIS B

Polysaccharide (1g; 50% carbohydrate) was dissolved in water (20 ml) and placed in a dialysis sac to which IR 120(H^+) resin (20 g.) was added. The open ended sac was suspended in a well stirred, heated, vessel containing distilled water (850 ml). The contents of the dialysis sac were well-stirred and the whole system was allowed to attain a temperature of ca. 70°C (see Figure 7a) (p 95). The dialysate was sampled for carbohydrate periodically by withdrawing an aliquot (1 ml) and when the solution indicated a concentration of about 50 μg/ml the dialysate was siphoned off, neutralized and replaced

with fresh distilled water. The whole process was repeated until the concentration of carbohydrate inside the dialysis sac was negligible.

During the autohydrolysis aliquots (ca. 20 ml) were removed separately from the first four dialysates, neutralised, evaporated to dryness and analysed by paper chromatography (solvent A; spray reagent (i)). The results of the analysis by visual estimation are shown in TABLE XIV.

SUGARS	R_{GAL} (Solvent A)	1st Aliquot	2nd Aliquot	3rd Aliquot	4th Aliquot
XYLOSE	1.3	++	++	++	++
ARABINOSE	1.2	+	+	++	++
GALACTOSE	1.0	++++	++++	++++	++++
IV	0.66 streak	+++	+++	+++	+++
V	0.22	++	++	++	T
VI	0.20	++	++	++	T
VII	0.06	+++	++	+	T

\+ = UNIT OF RELATIVE PROPORTION

T = TRACE

TABLE XIV

Continuous Autohydrolysis (System B)

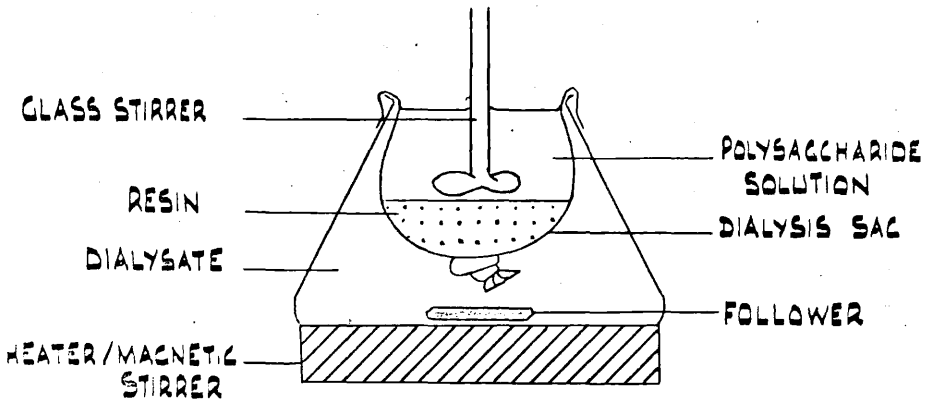


FIGURE 7 (a)

Sephadex G50 Gel Column Chromatographic Analysis of Polymer From Autohydrolysis B

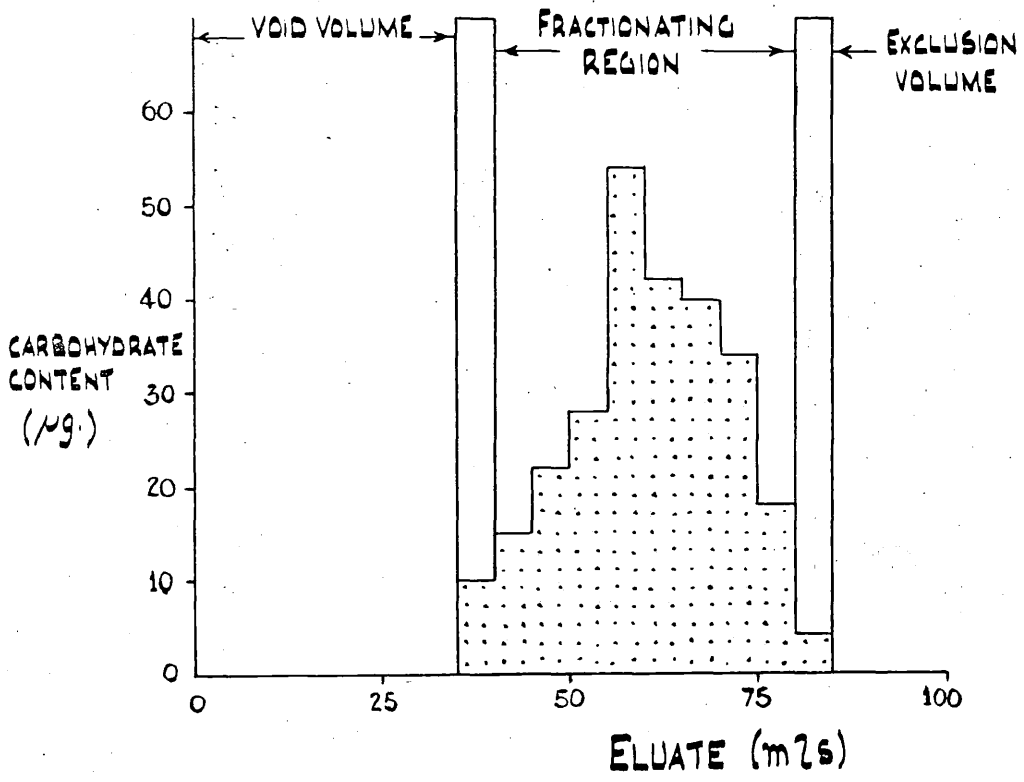


FIGURE 7 (b)

In view of their essential similarity the dialysates were combined and evaporated to a syrup, T, (2.27 g; carbohydrate 300 mg). This syrup was examined by paper chromatography with solvents A, B and E. Solvent A gave the best separation and was therefore chosen for preparative separation.

EXPERIMENT 20. SEPARATION OF THE HYDROLYSATE INTO COMPONENTS

A portion of T (0.75 g) was applied to Whatman No.17 paper and eluted with solvent (A) for 36 hours the monosaccharides (ca. 34 mg) being collected in a trough placed beneath the paper. The papers were removed and the bands containing carbohydrate detected by taking an imprint with a No.1 paper (G.M. 12) which was then developed with spray reagent (i). This system successfully detected fractions IV, V, and VI, but fraction VII could only be detected by developing side and centre strips (spray (i)). The components were eluted separately, reduced to small bulk, and freeze-dried. Considerable overlapping occurred with fractions V and VI and these were reseparated, firstly, by preparative ionophoresis (G.M. 14 buffer (i)), and then on Whatman No.1 paper in solvent H and isolated by freeze-drying after elution from the paper. Component VII was similarly purified by preparative ionophoresis (G.M.14 buffer (i)).

EXPERIMENT 21. ANALYSIS OF FRACTION IV

The syrup (ca. 16 mg.) R_{GAL} 0.66 (streak) (solvent A), 0.06 (streak) (solvent B); M_{GA} = 0, 0.6, 1.0 (Buffer (i) G.M.14) was separated into three components (IVA, IVB, and IVC) by preparative ionophoresis, isolated and freeze-dried.

IVA

The syrup (ca. 10 mg.) M_{GA} = 1, gave a brown spot with spray reagent (ii) and had the same chromatographic and ionophoretic mobilities as standard galactose-6-sulphate. Paper chromatography (solvents A and B; spray reagent (i) and (ii)) of a hydrolysate (G.M. 8(a)) revealed the presence of galactose only. The component had a DP of 1.

IVB

The syrup (ca. 4 mg.) M_{GA} = 0.6, gave a pink coloured spot with spray reagent (ii) had a DP of 2 and was paper chromatographically and ionophoretically homogeneous and had the same mobility as ~~arabinose~~ arabinobiose-3-sulphate. Paper chromatography (solvents A and B; spray reagent (i) and (ii)) of a hydrolysate (G.M. 8 (a)) revealed the presence of arabinose only.

IVC

The syrup (ca. 2 mg.) was neutral and gave a brown spot with spray reagent (ii) with the mobility of 1,3-galactobiose. On hydrolysis (G.M. 8(a)) and analysis by paper chromatography

(solvents A and B; spray reagent (i) and (ii) only galactose was revealed. The material had a DP of 2. Methylation, methanolysis, and examination by g.l.c.(columns 1 and 2) gave peaks with the same retention times as those of standard methyl 2,4,6-tri-O-methylgalactoside and methyl 2,3,4,6-tetra-O-methylgalactoside.

EXPERIMENT 22. ANALYSIS OF FRACTION V

A syrup (12 mg., sulphate; 36%); R_{GAL} = 0.22 (solvent A), 0.06 (solvent B) (M_{GA} = 1.20) was chromatographically and ionophoretically homogeneous in all systems tried giving an orange spot with spray reagent (ii). Paper chromatography (solvents A and B; spray reagent (i) and (ii)) of a hydrolysate (G.M. 8(a)) revealed the presence of galactose and arabinose. Reduction of the hydrolysate and analysis by g.l.c. as the TMS ether indicated a molar ratio of hexitol:pentitol of 1:1.6. The DP was 2.

Periodate oxidation of the reduced syrup by the micromethod of Belcher (G.M. 16) showed a periodate reduction of 2.1 moles periodate/disaccharide unit and a formaldehyde release of 0.86 moles disaccharide unit.

Methylation, methanolysis, and analysis by g.l.c. (Columns 1 and 2) indicated peaks with retention times the same as the following standards:-

methyl-2,3,4-tri-O-methylarabinoside

methyl-2,-O-methylarabinoside

methyl-2,3,4-tri-O-methylgalactoside

methyl-2,4-di-O-methylgalactoside

Reduction (G.M. 17) followed by hydrolysis (G.M. 8(a)) and examination by paper chromatography (solvents A and B; spray reagents (i) and (ii)) indicated the presence of galactose, arabinose, galactitol, and arabinitol.

An aliquot (10 mg) in water (0.2 ml) (prepared, as before, from a separate autohydrolysis experiment) was reduced (G.M. 17) for 12 hours. It was then treated with 2N-sodium hydroxide (2 ml) and the solution heated at 80°C for 7 hours with occasional additions of borohydride (3 x 10 mg). The resulting solution was cooled, neutralised with acetic acid, treated with IR 120(H^+) resin, filtered, evaporated to small bulk, treated with methanol (3 x 10 ml), evaporated to small bulk, neutralised (0.880 Ammonia) and evaporated to dryness. The syrup was examined by ionophoresis (G.M. 14 buffer (i)) and the presence of three compounds, one neutral and two acid (M_{GA} = 0.85 and 1.2 trace buffer (i)) were indicated. The neutral and major acid component (M_{GA} = 0.85) were separated by preparative ionophoresis (G.M. 14 buffer (i)), located (spray (i)), eluted off, reduced to small bulk and freeze-dried. The two materials were

hydrolysed (G.M. 8(a)) separately and examined by paper chromatography (solvents A, B and I; spray reagents (i) and (ii) which revealed the presence of:-

Neutral component:- galactose, arabinose, xylose, and galactitol.

Acidic component:- galactose, arabinose, xylose, galactitol, and arabinitol.

EXPERIMENT 23. ANALYSIS OF FRACTION VI

The syrup (20 mg) had, SO_4^{2-} 27%, DP 3, R_{GAL} 0.20 (solvent A), 0.06 (solvent B), M_{GA} 1.0 (buffer (i)), and gave a pinky brown spot (spray reagent (ii)). Chromatographic analysis (solvents A and B, sprays (i) and (ii)) of a hydrolysate revealed the presence of galactose and arabinose. Partial acid hydrolysis (N-sulphuric acid for 30 mins at 100°C) of a second aliquot gave galactose, arabinose, galactose-6-sulphate, and arabinose-3-sulphate (trace) [paper chromatography solvents A, B and C spray (ii) and ionophoresis (buffer (i)].

Reduction of the total hydrolysate and analysis by g.l.c. as the TMS derivatives gave a molar ratio of pentitol:hexitol of 1:2. Reduction of an aliquot followed by hydrolysis gave galactose and arabinitol [paper chromatography (solvents A and B; sprays (i) and (ii))].

Periodate oxidation of the reduced syrup (G.M. 16) gave a reduction of 2.2 moles periodate per trisaccharide unit liberating 0.75 mole formaldehyde per unit.

Methylation followed by methanolysis and examination by g.l.c. (columns 1 and 2) indicated the presence of methyl 2,3,4-tri-O-methylgalactoside, methyl 2,4-di-O-methylgalactoside, methyl 2,3-di-O-methylarabinoside, and methyl 2-O-methylarabinoside.

An aliquot (ca. 2 mg.) in water (0.5 ml) was reduced for 12 hours. The solution was divided into two equal portions and one half was treated with 2N-sodium hydroxide (2 ml) and the other treated with water (2 ml). Both were heated at 80-85°C for 7 hours with several additions of potassium borohydride (3 x 5 mg. each). The two solutions were cooled and an aliquot (0.2 ml) from each was made up to 2 ml. Both samples were placed in ice and resorcinol reagent (10 ml; composed of resorcinol 130 mg in 100 ml ethanol, added to 100 ml of 12M hydrochloric acid) was slowly added to each of the cold solutions with thorough mixing. The samples were then heated for 10 minutes at 80°C, cooled in ice for 1.5 minutes, and the optical density of the solution was measured at 550 mμ in a Unicam S.P. 500 spectrophotometer within 15 minutes. A difference of 0.22 units was observed between

blank and desulphated material indicating the presence of 3,6 anhydrogalactose formation.

EXPERIMENT 24. ANALYSIS OF FRACTION VII

The solid (ca. 40 mg), (found SO_4^{2-}, 18%) R_{GAL} 0.06 (solvent A) 0.06 (solvent B) was chromatographically homogeneous but gave a streak when examined by ionophoresis with a maxima (M_{GA} = 1). The material gave an orange coloured spot with spray reagent (ii). Paper chromatography (solvents A and B; spray reagents (i) and (ii)) of a hydrolysate revealed the presence of galactose and arabinose. Reduction of this hydrolysate and analysis by g.l.c. as the TMS derivative indicated a molar ratio of hexitol:pentitol of 1:4. The DP was 5.

Complete oxidation of the reduced oligosaccharide with 0.015M sodium metaperiodate (G.M. 16) resulted in the reduction of 4.9 moles periodate per pentasaccharide unit with the liberation of 1.8M of formaldehyde. An aliquot (ca. 2 mg) was treated with sodium metaperiodate for the same time as in the previous oxidation. The reaction was stopped by addition of ethylene glycol after which the oligoaldehyde was reduced with 2% potassium borohydride for 24 hours at 0°C. The solution was neutralised, hydrolysed with sulphuric acid (G.M. 8 (b)(ii))

deionised with Amberlite IR 120 (H^+) and IR 45 (OH^-) resins and evaporated to dryness. Examination by paper chromatography (solvents A and B; spray reagent (i) and (ii) revealed the presence of galactose, arabinose and glycerol. The galactose to arabinose ratio was approximately 1:2 (visual estimation from a paper chromatogram).

Partial acid hydrolysis of the material as for VI and analysis of the products by paper chromatography (solvents A and B; spray (ii)) and ionophoresis (buffer (i)) revealed the presence of arabinose, galactose, arabinose-3-sulphate, galactose-6-sulphate, and traces of oligomeric materials which had different mobilities to those of the oligomers V or VI.

Reduction of a sample (ca. 2mg.) followed by hydrolysis (G.M. 8(a)) and analysis by paper chromatography (solvents A and B; spray reagent (i) and (ii)) indicated the presence of arabinose, galactose, arabinitol, and galactitol (trace).

The material was methylated (G.M. 9) examined by thin layer chromatography on silica gel plates (G.M. 13; solvent Y) to ensure complete methylation, methanolysed (G.M. 9) and analysed by g.l.c. Peaks with retention times consistent with the following standards were identified.

methyl 2,3-di-O-methylarabinoside

" 2,3,4-tri-O-methylarabinoside

" 2,-O-methylarabinoside * MAJOR

" 3,-O-methylarabinoside

" 2,4-di-O-methylgalactoside

" 2,3,5,6-tetra-O-methylgalactoside

Attempts to show the presence of 3,6 anhydrogalactose with resorcinol reagent after small scale alkali desulphation, as for component VI, failed.

The material (ca. 20 mg.) was reduced; and treated with 2-N sodium hydroxide in the same way as for fraction V (p.99). Ionophoresis (buffer (i)) of the derived material indicated an acid and a neutral component in approximately equal quantities. These were separated by preparative ionophoresis. Hydrolysis of both acid and neutral components and examination by paper chromatography (solvents A and B; spray reagents (i) and (ii)) revealed the presence of galactose, arabinose, and xylose. Both acid and neutral fractions were methylated separately and examination by thin layer chromatography (G.M. 13; solvent Y) indicated complete methylation. Both products were methanolysed and examined by g.l.c. (columns 1 and 2). For the neutral component peaks consistent with the following standards were observed.

methyl 2,3,4-tri-O-methylarabinoside

methyl 2,3-di-O-methylarabinoside

methyl 3,-O-methylarabinoside

methyl 2,3,4-tri-O-methylxyloside (trace)

methyl 2,3-di-O-methylxyloside

methyl 2,3,5,6-tetra-O-methylgalactoside

For the acidic component the xylose and arabinose derivatives were the same but the major galactose derivative was methyl-2,4-di-O-methylgalactoside.

EXPERIMENT 25. ANALYSIS OF THE MONOSACCHARIDE FRACTION FOR OLIGOMERS

The neutral syrup (ca. 34 mg.) containing the monosaccharide fraction, glycerol and any oligomeric materials was applied to a Sephadex G.10 column (2 cm x 20), previously calibrated with blue dextran to find the void volume and with glucose and sucrose to determine the hexose and disaccharide elution positions. The column was eluted with water and the eluate collected in 2 ml. aliquots each being examined for carbohydrate content. Carbohydrate was only detected in that eluate assigned to the monosaccharide region. No oligomeric material was present in this fraction. The monosaccharide

component was evaporated and reseparated by paper chromatography (solvent 4). This yielded galactose (26 mg) and xylose (8 mg).

EXPERIMENT 26. ANALYSIS OF A POLYMER OBTAINED FROM AUTOHYDROLYSIS B

During the partial autohydrolysis B (EXPT.19 p.93) it was noticed that the pH rose after the first ten hours of reaction. Polysaccharide (1 g) was autohydrolysed therefore as previously. The pH was observed and again an increase occurred after 10 hours. The resin was filtered off at once using glass wool. The acidic filtrate contained a fine sediment not retained by the filter pad and this was separated by centrifugation. Both the solid suspended in water and the solution were neutralised (0.880 Ammonia), evaporated to small bulk, and freeze-dried. (Acid soluble = 196 mg; carbohydrate content 65%; sulphate content 26%. Acid insoluble = 121 mg, carbohydrate content 6%; sulphate 18%). Both materials were hydrolysed (G.M. 8(a)) and examined by paper chromatography (solvents A and B; spray reagent (i) and (ii)) which revealed the following sugars in the proportions given below (visual estimation) in TABLE XV.

	GALACTOSE	ARABINOSE	XYLOSE	RHAMNOSE
INSOLUBLE FRACTION	+++	+++	-	-
SOLUBLE FRACTION	++	++++	+	+

TABLE XV

An approximate value for the average molecular weight of the water soluble component was determined using a Sephadex G.50 column (90 x 1 cm) eluted with 0.5M-sodium sulphate. The column was calibrated with blue dextran and 0.005M-sucrose solution to determine the void and exclusion volumes.

The sample of polymer (ca. 3 mg.) was applied to the column in 1M-sodium sulphate solution and eluted with 0.5M-sodium sulphate. The eluate was collected in 5 ml samples and each fraction was examined for carbohydrate content. The result of the analysis is shown in Figure 7(b)(p95). From the position of the maxima the average D.P. was calculated to be 16-22 units.

Attempts to separate the polymer into fractions by DEAE-cellulose paper chromatography failed as did ionophoresis, the former showing slight separation under the buffer systems used, and the latter giving only a streak from just ahead of the baseline to $M_{GA} = 1.25$.

EXPERIMENT 27. PARTIAL ACETOLYSIS OF THE DESULPHATED POLYSACCHARIDE

To desulphated polysaccharide (1 g. 70% carbohydrate, 10.6% sulphate, preparation see expt. 30) a solution (20 ml) of acetic anhydride and analar concentrated sulphuric acid (100:9 v/v) at 0°C, was added slowly during a period of 1 hour. The solution was then placed in an incubator at 30°C for 30 hours.[87]

The reaction mixture was then diluted with iced water (40 ml) and neutralised by addition of solid sodium bicarbonate. The aqueous solution was extracted with chloroform (3 x 500 ml), the combined chloroform layers extracted with dilute (2%) aqueous sodium bicarbonate solution, and finally dried over anhydrous sodium sulphate overnight. The clarified solution was filtered and evaporated to dryness. Dry methanol was added to the residue, filtered, and the filtrate evaporated. The residue was taken up in dry methanol (10 ml) and a pellet (ca. 0.2 $cm.^3$) of sodium metal dissolved in the solution, almost immediately a yellow precipitate formed. The solution was left at room temperature for 8 hours after which it was poured into water (ca. 20 ml) and Amberlite IR 120(H^+) resin added. After an hour the solution was found to be neutral and was evaporated to a syrup (100 mg.)(A_1).

EXPERIMENT 28. EXAMINATION AND SEPARATION OF OLIGOMERIC COMPONENTS FROM PARTIAL ACETOLYSIS

The syrup (100 mg)(A_1) was examined by paper chromatography (solvents A and B; spray reagent (i) which revealed a complex mixture of components from R_{GAL} 1.5-0.40. It was separated into monosaccharide and oligosaccharide fractions by charcoal-Celite column chromatography (see Expt. 17).

The syrup was applied to the column which was then eluted with water. The eluant was periodically tested for carbohydrate content until this figure was negligible (ca. 2L). The column was then eluted with 20% ethanol (ca. 4L). This alcoholic eluate was evaporated to a syrup (5-10 mg.).

The derived syrup was separated into six components on Whatman No.1 paper by elution for 36 hours with solvent A.

EXPERIMENT 29. ANALYSIS OF OLIGOMERS FROM PARTIAL ACETOLYSIS I

An aliquot of syrup (total ca. 0.46 mg.), R_{GAL} 1.42 (solvent A), DP 2, was hydrolysed (G.M. 8(a)) and paper chromatographic analysis (solvents A and B; spray reagents (i) and (ii)) revealed the presence of xylose and rhamnose.

Methylation, methanolysis, and analysis by g.l.c. (columns 1 and 2) indicated the presence of methyl 2,3,4-tri-O-methyl-

rhamnoside and methyl 2,3-di-O-methylxyloside.

II

A syrup (ca. 0.44 mg.) R_{GAL} 1.24 (solvent A) had a DP of 2. Examination of a hydrolysate (G.M. 8(a)) by paper chromatography (solvents A and B spray (i) and (ii)) revealed the presence of arabinose (major) and rhamnose.

Methylation, methanolysis, and analysis by g.l.c. (columns 1 and 2) gave peaks consistent with methyl 2,3,4-tri-O-methyl-rhamnoside, methyl 2,3-di-O-methylrhamnoside, methyl 2,3,4-tri-O-methylarabinoside, and methyl 2,3-di-O-methyl-arabinoside.

III

A syrup (ca. 0.31 mg.), R_{GAL} = 1.14 (solvent A), had a DP of 2.33. Examination of a hydrolysate (G.M. 8 (a)) by paper chromatography (solvents A and B; spray reagent (i) and (ii)) revealed the presence of galactose and rhamnose.

Methylation, methanolysis, and analysis by g.l.c. (columns 1 and 2) gave peaks consistent with the properties of methyl 2,3,4-tri-O-methylrhamnoside, methyl 2,4-di-O-methylrhamnoside and methyl 2,3,4,6-tetra-O-methylgalactoside.

IV

A syrup (ca. 0.46 mg.) R_{GAL} = 1.00, had a DP of 2.69. Examination of a hydrolysate (G.M. 8(a)) by paper chromatography (solvents A and B; spray reagents (i) and (ii))

revealed the presence of galactose and rhamnose.

Methylation, methanolysis, and g.l.c. examination indicated the presence of methyl 2,4-di-O-methylrhamnoside, methyl 2,3,4-tri-O-methylrhamnoside, and methyl 2,3,4,6-tetra-O-methylgalactoside.

V

A syrup (ca. 0.44 mg), R_{GAL} = 0.82 had a DP of 3. Examination of a hydrolysate (G.M. 8 (a)) by paper chromatography (solvents A and B, spray reagents (i) and (ii)) revealed the presence of galactose and rhamnose.

Methylation, methanolysis, and g.l.c. examination indicated the presence of methyl 2,3,4-tri-O-methylrhamnoside, methyl 2,4-di-O-methylrhamnoside, and methyl 2,3,4-tri-O-methylgalactoside.

VI

A syrup (ca. 0.37 mg), R_{GAL} = 0,43, had a DP of 2. Examination of a hydrolysate (G.M. 8 (a)) by paper chromatography (solvents A and B, spray reagents (i) and (ii)) revealed the presence of galactose with a trace of rhamnose.

Methylation, methanolysis, and g.l.c. examination indicated the presence of methyl 2,3,4,6-tetra-O-methylgalactoside and methyl 2,3,4-tri-O-methylgalactoside.

PART IV

DESULPHATION AND METHYLATION STUDIES ON THE HOT-WATER-SOLUBLE POLYSACCHARIDE EXTRACT FROM *CLADOPHORA RUPESTRIS*

EXPERIMENT 30. SMALL SCALE DESULPHATION STUDIES

(a)

The polysaccharide (400 mg., 50% carbohydrate) was treated with methanol containing 1% hydrogen chloride (80 ml) and the suspension shaken for 24 hours at room temperature. The solid residue was recovered by centrifugation and the supernatant discarded. Half the residue was treated with 1% hydrogen chloride (40 ml) for a further 24 hours and the residue separated as before. The two residues were separately suspended in water, neutralised with dilute ammonia, dialysed extensively against distilled water, reduced to small bulk and freeze-dried. The results are given in TABLE XVI.

SAMPLE	WEIGHT RECOVERED	CARBOHYDRATE	SULPHATE*
0 hrs contact with 1% MeOH-HCl	200 mg.	50%	22%
24 hrs contact with 1% MeOH-HCl	120 mg.	34%	22%
48 hrs contact with 1% MeOH-HCl	82 mg.	21%	40%

* Based on carbohydrate content.

TABLE XVI

(b)

Three aliquots of polysaccharide (200 mg. each) were treated with 0.35% methanolic hydrogen chloride at room temperature, the first for 24 hours using 40 ml of reagent, the second for 48 hrs (2 x 40 ml), and the third for 96 hours (4 x 40 ml), and the residual polysaccharides recovered as before. The results are shown in TABLE XVII.

SAMPLE	WEIGHT RECOVERED	CARBOHYDRATE	SULPHATE*
24 hrs contact with 0.35% MeOH/HCl	99.0 mg.	70%	16.5%
48 hrs contact with 0.35% MeOH/HCl	90.0 mg.	70%	10.4%
96 hrs contact with 0.35% MeOH/HCl	34.0 mg.	33%	18.0%

* Based on carbohydrate content

TABLE XVII

EXPERIMENT 31. LARGE SCALE DESULPHATION OF POLYSACCHARIDE

Polysaccharide (9 g., 50% carbohydrate) was partially desulphated by treating with 0.35% methanolic hydrogen chloride (2000 ml x 2) for two separate periods of 24 hours. The solid residue was washed twice with methanol and, after suspension in water, neutralised with dilute ammonia solution. The suspension was dialysed extensively against distilled water, reduced to small bulk, and freeze-dried (4.46 g., 70% carbohydrate, 10.6% sulphate).

A portion of this product (ca. 5 mg.) was hydrolysed (G.M. 8 (a)) and the hydrolysate examined by paper chromatography (solvents A and B, syrup (i) and (ii)) which revealed the presence of galactose, arabinose, xylose, and rhamnose, g.l.c. analysis as the TMS derivative confirmed the presence of these sugars. The relative proportions were calculated and are shown in TABLE XVIII

	GALACTOSE	ARABINOSE	XYLOSE	RHAMNOSE
ORIGINAL POLYSACCHARIDE	1.00	0.84	0.37	TRACE
DESULPHATED "	1.00	1.96	0.44	TRACE

TABLE XVIII

EXPERIMENT 32. METHYLATION OF DESULPHATED MATERIAL USING SRIVASTAVA'S METHOD

Polysaccharide (4.46 g., 70% carbohydrate, 10.6% sulphate) was dissolved in dimethyl sulphoxide (120 ml) and methylated under nitrogen with dimethyl sulphate (88 ml) and sodium hydroxide (108 g.), added in portions with vigorous stirring during 6 hours. The mixture was maintained at 0°C during the first two additions. Stirring was continued overnight (ca. 12 hours) and then the solution was treated with concentrated ammonia solution (60 ml) to destroy excess dimethyl sulphate. The reaction mixture was neutralised with

15N-sulphuric acid and then dialysed extensively against frequently changed distilled water.

The non-dialysable component was evaporated to small bulk and placed in a liquid-liquid extractor using chloroform as the extracting agent (ca. 500 ml). The system was left 6 hours after which time the aqueous component was recovered and the chloroform soluble fraction evaporated to dryness. No residue remained.

The aqueous component was dialysed for a further period and then concentrated and freeze-dried to a cream coloured solid. (3.34 g., Found: OMe, 4%).

EXPERIMENT 33. METHYLATION OF PARTIALLY METHYLATED POLYSACCHARIDE BY HAWORTH'S METHOD

The partially methylated polysaccharide (3.34 g.) was suspended in water (60 ml) and nitrogen bubbled vigorously through the solution. Dimethyl sulphate (60 ml) and 30% sodium hydroxide solution (80 ml) were added slowly over a period of 6 hours with efficient stirring. The stirring was continued for a further 8 hours. The whole procedure was repeated three times at room temperature.

The reaction was halted by addition of concentrated ammonia solution (200 ml) and the solution neutralised with

15N-sulphuric acid. The mixture was dialysed extensively against distilled water, concentrated to small bulk and extracted with chloroform as previously. The aqueous component was recovered, dialysed, evaporated to small bulk and freeze-dried to an off-white solid (B) (3.5 g Found: OMe 15.7%). The chloroform was evaporated to dryness and yielded a yellow gum which on subsequent suspension in water and freeze-drying yielded a white solid (A) (250 mg, Found: OMe 21.3%).

EXPERIMENT 34. METHYLATION OF PARTIALLY METHYLATED POLYSACCHARIDE BY KUHN'S AND PURDIE'S PROCEDURE

The partially methylated component B (3.5 g., OMe 15.7%) was dissolved in redistilled dimethyl formamide (60 ml) and to this solution was added redistilled methyl iodide (100 ml) followed by dry silver oxide (5 g.). The mixture was shaken for 18 hours at room temperature, the silver salts removed by filtration and well-washed with chloroform. The combined washings and filtrate were evaporated to dryness under vacuum (0.1 - 0.5 mm of mercury), (3.2 g.)(C).

The material (C) and (A) (250 mg. OMe 21.3%) were combined and dissolved in methyl iodide (100 ml). Silver oxide

(7 g.) was added and the mixture refluxed for 6 hours. The separated silver salts were extracted with chloroform/dimethyl formamide solution (1:1 v/v) and the extract evaporated to dryness (D). The solution from the Purdie reaction was evaporated to dryness, suspended in water and freeze-dried (E) (1.3 g., OMe 28.4%).

The solid (D) was given a second Kuhn methylation as before to yield a freeze-dried solid (0.40 g., OMe 20%).

EXPERIMENT 35. FRACTIONATION OF METHYLATED COMPONENT (E)

The methylated polysaccharide (1.3 g.,OMe 28.4%) was extracted sequentially with light petroleum (b.pt 60-80°)/chloroform solvents (mixed 80:20, 70:30, 60:40, 50:50, 0:100 v/v) under reflux for 15 minutes with each solution. Each of the extracts was evaporated, suspended in water and freeze-dried, (see TABLE XIX).

EXTRACTANT		WEIGHT	% METHOXYL	% METHOXYL (CORRECTED)*	% SULPHATE
LIGHT PETROLEUM	CHLOROFORM				
(P)80 :	20	149 mg.	26.6%	33%	10.6%
(Q)70 :	30	168 mg.	35.4	39.7	5.6
(R)60 :	40	631 mg.	31.7	36.7	2.3
(S)50 :	50	221 mg.	30.9	35.7	3.2
(T) 0 :	100	89 mg.	25.3	30.1	5.1

* Corrected for sulphate, nitrogen and Ash.

TABLE XIX

EXPERIMENT 36. SMALL SCALE HYDROLYSIS OF METHYLATED FRACTIONS (P)-(T)

An aliquot (ca. 2-5 mg) of each of the methylated fractions (P-T) was hydrolysed (G.M. 8 (a)) and examined by paper chromatography (solvent D and G spray reagent (ii)). This showed the same pattern for all fractions with very little differences in relative proportions (visual estimation), as shown below in TABLE XX.

VISUAL ESTIMATION	R_G (SOLVENT G)	R_G (SOLVENT D)	COLOUR
++	0.96	0.99	Pink
TRACE	0.88	0.89	Yellow Brown
TRACE	0.80	0.82	Pink
++++	0.71	0.75	Pink/Brown
++	0.54	0.48	Pink
+	0.46	0.42	Yellow Brown
++	0.35	0.32	Pink
TRACE	0.20	0.10	Pink
TRACE	0.05	0.05	Brown

TABLE XX

EXPERIMENT 37. LARGE SCALE HYDROLYSIS OF METHYLATED POLYSACCHARIDE AND SEPARATION OF COMPONENTS BY CELLULOSE COLUMN AND PAPER CHROMATOGRAPHY

Methylated polysaccharide (800 mg.) [composed of fraction (Q) (70 mg.), fraction (R) (600 mg.) and fraction (S) (130 mg.)], was hydrolysed (G.M. 8 (a)) and the hydrolysate evaporated to a thick syrup. A column (60 cm x 3.5 cm) was packed, under pressure, with Whatman Chromedia C.F. 11 cellulose powder (100 g) using acetone as the solvent. The packed column was washed with acetone (2L), acetone-n-butanol (2L), n-butanol saturated with water (2L), and, finally, light petroleum (b.pt. 100-120°)-n-butanol (7:3 v/v) saturated with water (4L).

The mixture of methylated derivatives dissolved in water (ca. 2 ml) was applied to the column and eluted with light petroleum (b.pt. 100-120°)-n-butanol (7:3 v/v) saturated with water. Fractions of the eluate (25 ml) were collected and every 10th tube analysed by paper chromatography (solvent D, spray (ii)). The solvent was changed to light petroleum b.pt. 100-120°)-n-butanol (1:1v/v) saturated with water at tube 500 and to n-butanol saturated with water at tube 850 during the elution. This gave only partial separation and the following fractions were isolated (see TABLE XXI).

TUBE NO.	FRACTION PRESENT
50-70	I
71-159	II III IV V
160-490	V VI
491-820	VII VIII
821-859	VII(Trace) VIII IX(Trace)
860-900	IX
901-940	IX X
941-1020	X

TABLE XXI

Each of the fractions was badly contaminated with waxy material from the column.

The fractions containing a number of components were reseparated on Whatman No.3 papers by elution with solvent D. The eluant was allowed to reach the bottom of the paper in every instance and then the paper was air dried for one hour. The papers containing the sugars from tube 160-490 were developed a second time and those with material from tubes 491-859 were developed three times for 12, 8, and 8 hours respectively, before location and elution of the different methylated sugars.

EXPERIMENT 38. ANALYSIS OF PURIFIED FRACTIONS

FRACTION I

A syrup, 10 mg., $[\alpha]_D$ = +10° (cf. 2,3,4-tri-O-methylxylose $[\alpha]_D$ = +18° [88]) which slowly crystallised, and had a m.p. and mixed m.p. of 86° identical to that of authentic 2,3,4-tri-O-methylxylose. It gave a single spot on a paper chromatograph with a mobility identical to authentic 2,3,4-tri-O-methylxylose. Demethylation (G.M. 11) gave only xylose. Analysis of the sample by g.l.c. as the methylglycoside gave peaks with retention times identical to authentic methyl 2,3,4-tri-O-methyl-D-xyloside on columns (1) and (2).

FRACTION II

A syrup, 6.5 mg., was a mixture. Demethylation (G.M.11) of a portion of this syrup (ca. 3 mg.) (paper chromatography solvents A and B, spray (ii)) revealed the presence of galactose (major) and xylose. Analysis of the derived glycosides by g.l.c. (columns 1 and 2) indicated the presence of peaks identical with those of standard methyl 2,3,4-tri-O-methyl-xyloside and methyl 2,3,5,6-tetra-O-methylgalactoside.

FRACTION III

A syrup, 10.0 mg., was a mixture which on demethylation (G.M. 11) revealed the presence of rhamnose (major), galactose, and arabinose (paper chromatography solvents A and B spray reagent (ii)). The syrup (ca. 3 mg.) was converted

to the methyl glycoside and examined by g.l.c. (columns 1 and 2). The presence of peaks with the same retention times as those of standard,

	RETENTION TIMES	
	COL(1) R_{TMG}	COL(2) R_{TMG}
methyl 2,4-di-O-methylrhamnoside	1.12	0.66
" 3,4-di-O-methylrhamnoside	1.0,1.71	0.57
" 2,3,4,6-tetra-O-methylgalactoside	1.99,1.86(sh)	1.61,1.52(sh)
" 2,3,5-tri-O-methylgalactoside	3.9	2.1
" 2,3,4-tri-O-methylarabinoside	1.0	0.85

where (sh) = shoulder

were observed.

FRACTION IV

A syrup, 13.1 mg., which streaked on a paper chromatogram (solvent D, R_G 0.86-0.79). The syrup (ca. 3mg.) was demethylated (G.M. 11) and paper chromatography (solvents A and B, spray reagent (ii)) revealed the presence of galactose, xylose, rhamnose, and arabinose. The fraction (ca. 5 mg.) was converted to the methyl glycosides and examined by g.l.c. (columns 1 and 2). Peaks with the same retention times as those of the following standard sugars were obtained.

	RETENTION TIMES	
	COL(1) R_{THG}	COL(2) R_{THG}
methyl 3,4-di-O-methylrhamnoside	1.01, 1.71	0.57
" 2,3,5-tri-O-methylgalactoside	3.9	2.1
" 2,3-di-O-methylxyloside	1.67, 1.79	0.76, 0.96
" 2,3-di-O-methylarabinoside	1.64, 1.78	0.66,1.0,0.85

FRACTION V

A syrup, 135 mg., $[\alpha]_D$ = + 76° (cf. 2,3-di-O-methylarabinose $[\alpha]_D$ = + 101° [88]) which failed to crystallise although it was chromatographically homogeneous (solvents A and B) and had the mobility and colour (spray reagent (ii)) of a dimethyl pentose. Demethylation (G.M. 11) of a sample (ca. 3 mg.) and paper chromatography (solvents A and B, spray reagent (ii)) of the derived syrup, revealed the presence of arabinose only.

The syrup (ca. 5 mg.) was converted to the methyl glycoside (G.M. 9) and analysed by g.l.c. (columns 1 and 2) and gave peaks consistent with those of standard methyl 2,3-di-O-methylarabinoside. A further sample of the syrup (ca. 3 mg.) was reduced to the alcohol and acetylated (G.M. 15 (c)) and examined by g.l.c. (column 4), peaks identical to those of 2,3-di-O-methylarabinitol 1,4,5-triacetate, were obtained.

The syrup (50 mg.) was converted to the N-phenyl-glycosylamine (anilide) by refluxing with 5% methanolic aniline (2 ml) for 4 hours. The resulting solution was allowed to evaporate at room temperature to a syrup. This was extracted with ether (1-2 ml) and, to the extract, light petroleum (b.pt. 0 - 40) (10 ml) was added resulting in a faintly turbid solution. The cloudy solution was left at -10°C for 12 hours. The derived crystals were washed quickly with cold acetone (-10°C) and dried (m.pt. = 137°C) (cf. 2,3-di-O-methyl-L-arabinose N-phenyl-glycosylamine 139°C [89]).

FRACTION VI

A syrup, 16.5 mg., was chromatographically homogeneous in solvents D and G and had a mobility and colour (spray reagent (ii)) consistent with a trimethyl-galactose derivative. A sample (ca. 3 mg) was demethylated (G.M. 11) and analysed by paper chromatography (solvents A and B, spray reagent (ii)) revealing the presence of galactose with a trace quantity of arabinose. A second sample (ca. 3 mg) was converted to the methyl glycoside (G.M. 9) and analysed by g.l.c. (columns 1 and 2), peaks with retention times identical to those of standard methyl 2,3,4-tri-O-methyl-galactoside were obtained together with tiny peaks of

methyl 2,3 di-O-methylarabinoside.

The remaining material (ca. 10 mg.) was converted to the N-phenylglycosylamine derivative as for fraction V and the recrystallised material melted (m.pt. 162.5°C (sharp); cf. 2,3,4-tri-O-methylgalactose N-phenylglycosylamine m.pt. = 164° [90]).

FRACTION VII

A syrup, 45.0 mg., was homogeneous in solvents D and G and had the mobility and colour (spray (ii)) of standard 2-O-methylarabinose. Demethylation (G.M. 11) of a sample (ca. 3 mg.) and subsequent examination by paper chromatography (solvents A and B, spray reagent (ii)) revealed the presence of arabinose. The methylated derivative was converted to the methyl glycoside and examined by g.l.c. (columns 1 and 2) and peaks with identical retention times to those of standard methyl 2-O-methylarabinoside were obtained.

The methylated derivative gave a grey-green spot on a paper chromatogram (solvent D) with diphenylamine/phosphoric acid reagent.[91] Treatment of a similarly run chromatogram with triphenyltetrazolium hydroxide[92] failed to produce any colouration. Both the chromatograms contained standards which reacted positively with these reagents.

The syrup (ca. 35 mg) was converted to the mono-isopropylidene derivative by dissolving in acetone (1 ml) containing 0.5% concentrated hydrochloric acid and shaking for 2 hours. The reaction mixture was neutralised by being poured into 2.5% potassium bicarbonate solution (2 ml). This aqueous solution was extracted with chloroform, (1 x 10ml and 2 x 5), then with benzene (2 x 10 ml), and the extracts were combined and evaporated. The residue was taken up in ether (1-2 ml) and light petroleum (b.pt.20 - 40°) added until the solution became turbid. After leaving 12 hours at -10°C an oil had separated which after decanting the supernatant and washing with ether (0.1 ml) spontaneously crystallised. (M.pt. = 115-118°C; cf. 3,4-isopropylidene-2-O-methyl-L-arabinose m.pt. 116-118°).[93]

FRACTION VIII

A syrup, (21 mg) was an inseparable mixture of two components (solvents D and G), the major material had the colour and mobility of a monomethyl pentose. The fraction (ca. 3mg) was demethylated (G.M. 11) and paper chromatographic analysis (solvents A and B spray reagent (ii)) revealed the presence of arabinose (major) and galactose. Samples of the methylated derivative were examined by paper

chromatography (solvent D) and sprayed with diphenylamine/ phosphoric acid and triphenyltetrazolium hydroxide, the former reagent produced a grey-green spot (for the pentose derivative) and the latter reagent produced a blue spot.

The syrup (ca. 3 mg.) was converted to the methyl glycoside (G.M. 9) and examined by g.l.c. (columns 1 and 2) and gave peaks with retention times consistent with those of standard methyl 3-O-methylarabinoside and methyl 2,4 di-O-methylgalactoside (column 2 only).

All attempts to prepare crystalline derivatives of the mono- methyl pentose failed.

FRACTION IX

A syrup (5 mg.), with a positive rotation (ca. 60-70°) was chromatographically homogeneous (solvents A, B, D, and G), and had the mobility and colour (spray reagent (ii)) of arabinose. Analysis (ca. 1 mg.) by g.l.c. as the trimethylsilyl ether (columns 1 and 3) gave peaks identical to those given by standard arabinose.

The remainder of the material was treated with activated charcoal in hot aqueous solution, filtered, and the filtrate evaporated to dryness. The syrup slowly crystallised.

FRACTION X

A syrup, 5 mg, with a positive rotation, was chromatographically homogeneous (solvents A, B, D, and G) and had the mobility and colour of galactose. Conversion of a sample (1 mg) to the trimethylsilyl ether (G.M. 15 B(ii)) and analysis by g.l.c. (columns 1 and 3) confirmed that observation. A warm aqueous solution of the remaining syrup was decolourised with activated charcoal, after filtration, the clear liquid was reduced to a syrup which has so far failed to crystallise.

EXPERIMENT 39. DETERMINATION OF THE RELATIVE PROPORTIONS OF THE MAJOR METHYLATED SUGAR DERIVATIVES

Methylated polysaccharide (Q; OMe 40.3%, ca. 5 mg) was converted to the methylglycosides (G.M. 9) and examined by g.l.c. (column 2) using a Perkin-Elmer chromatograph and flame ionisation detector. The chromatogram was examined, firstly, to classify the peaks, and secondly, to measure the area under the peaks associated with each sugar derivative using the normal triangulation procedure. Using the following formulation[94] the approximate value of the detector factor for each component was calculated.

$$f' = \frac{M_i}{n \times 12}$$

where f' = approximate detector factor
M_i = Mol. wt. of substance i
n = No. of carbon atoms in i

The following table (TABLE XXII) gives the calculated details.

f'	Area	f' x Area	R.M.P.	Substance
1.97	43.00	84.71	51.8	methyl 2,3-di-O-methyl-arabinoside
2.08	3.83	7.96	5.3	methyl 2-O-methylarabino-side
2.08	2.66	5.53	3.7	methyl 3-O-methylarabino-side
1.96	4.60	9.02	4.4	methyl 2,3,4-tri-O-methyl-galactoside
2.04	0.93	1.90	1.0	methyl 2,4-di-O-methyl-galactoside
1.88	5.49	9.94	6.0	methyl 2,3,4-tri-O-methyl-xyloside

R.M.P. = Relative Molar Proportion

TABLE XXII

The methanolysed material was converted to the trimethylsilyl ethers and examined by g.l.c. using the same machine, detector and column. The chromatogram was analysed for the proportions of unmethylated galactose and arabinose. They were found to be negligible, within the accuracy of the method.

DISCUSSION

The hot water-soluble polysaccharide from Cladophora rupestris has been extracted by a route combining the methods of both Fisher and Percival[56] and Mackie and Percival[55] with, in addition, the use of α-amylase to remove the starch-type polymer. The yield of polymer using this system was 5%, which compares favourably with an extract which was not de-proteinised and in which a yield of 8% was obtained. The implication is that during this step, carried out in an acid medium, material is lost. Fisher[56] has shown that a small change in relative proportions of the sugars does indeed occur during this treatment (see TABLE XXIV).

RELATIVE PROPORTIONS				POLYMER
GALACTOSE	ARABINOSE	XYLOSE	RHAMNOSE	
3.1	3.2	1.0	TRACE	INITIAL POLYMER
2.8	3.7	1.0	TRACE	AFTER TCA TREATMENT

TABLE XXIV

The significance of this loss of galactose will be discussed later, suffice it to say that some degradation of the molecule is inevitable in order that a more highly purified extract can be obtained.

EXAMINATION OF THE HOMOGENEITY OF THE EXTRACT

In previous studies a large variety of techniques have been tried to fractionate this extract into a series of protein-free homopolymers. These have included the formation of copper complexes[56] and column chromatography on DEAE-cellulose and DEAE-Sephadex.[55] Three further methods have been investigated in the present studies.

GEL FILTRATION

Gel filtration of the extract on Seravac's Sagavac 6F., which is a crosslinked agarose based gel, was carried out. For many years gel column chromatography has been used for separation of polymeric species mainly of a proteinaceous nature.[96] However, instances are known where these systems have been used for analysis of carbohydrate polymers and oligomers. Anderson *et al.* in their work on *Acacia* gums have used the crosslinked polyacrylamide gel Biogel P200 extensively for fractionation of variously degraded polymers.[97,98]

The use of the Sephadex range, crosslinked dextrans, has mainly been restricted to the separation of oligomers or to the desalting of carbohydrate solutions.[96] However, there are a few instances where this gel system has been used for purification of polysaccharide extracts. Barker *et al.*[99] used a Sephadex G-200 column eluted with pH 7 phosphate

buffer for purification of hyaluronic acid produced by the action of pronase on human synovial fluid. The Birmingham school[100] have also used G-200 as a stage in the purification of a bacterial polysaccharide (Pneumococcus type II) using 1M-sodium chloride as eluent. Wicken and Baddiley[101] purified a teichoic acid type extract from a Streptococcus specie on G-75. Finally separation of high molecular weight polysaccharides have been reported by Hummel and Smith using Sepharose, the agarose gel manufactured by Pharmacia.

Seravac's Sagavac 6F was used in these studies because it had been found previously that the polymer was eluted in the void volume of both Biogel P200 and Sephadex G-200 and the theoretical data for Sagavac implied that its fractionating range was an order larger than these.

When the polymer was analysed on a Sagavac column it was found to elute completely in the region between the void volume and the exclusion volume. The shape of the elution curve (see page 61) was the same on repeated elution from the column. It was possible to divide this into three regions (A, B and C) and chromatographic analysis of hydrolysates from each of these regions showed no significant difference in their pattern. No apparent enrichment of

xylose could be detected in any fraction in contrast to results obtained previously using D.E.A.E. Sephadex A50.[55] The elution pattern in the present experiment indicates a wide molecular weight dispersity in the polymer, with reasonably constant monosaccharide composition.

The effluent from the column was also examined at 280 mμ on a spectrophotometer to determine whether residual protein had been separated from the polysaccharide on the column. A large peak of UV sensitive material was observed behind the polysaccharide region and hence it was supposed that a separation had been achieved. However the recovered polysaccharide on analysis for nitrogen content showed no decrease in this figure. Re-examination of the 280 mμ readings indicated a very small peak associated with the polysaccharide band and this is considered to represent the protein present. The nature of the larger peak can only be a source of speculation but the eluent associated with this material was yellow in colour and present preliminary studies have indicated the presence of phenolic species in the extract. It may well be that this peak does represent some form of polyphenolic compound, but until more intensive studies are carried out it must remain only a tentative conclusion.

GEL ELECTROPHORESIS

The second technique used for homogeneity studies was electrophoresis on a polyacrylamide gel column. This has been used mainly in the field of protein chemistry.[102] The technique involves separation on the basis of two distinct processes:-

(a) by charge, (the result of pH conditions)

(b) by size and shape (the effect of interaction with the crosslinked polyacrylamide matrix).

In the case of the present polysaccharide the overall charge on the molecule is little affected in the pH range 3-10 at which the analyses are run, hence this material will be mainly fractionated by process (b). However any protein not strongly associated with the polysaccharide has a charge affected by pH conditions as well as an interaction with the column matrix. In practice the extract moved as a single wide band (3 mm). This would seem to indicate that both materials were voided from the column matrix (see gel chromatography) and that the band broadening was an effect of charge dispersity already seen by ionophoresis. The fact that protein and carbohydrate are coincidental seems evidence that they are covalently linked.

ULTRA-FILTRATION

The extract was subjected to examination by ultra-filtration. The makers of the apparatus produce filter pads, which, they claim, have pore diameters of a strictly limited size range of which they quote the average. Molecules of varying size and shape will be fractionated according to their ability to pass through the pores. The solids retained on the filter pads, 250mμ 150mμ 20-30mμ and 5mμ, from a solution of the starch-containing polysaccharide, which had been passed through the membranes under high pressure, were hydrolysed and examined chromatographically. All of them, with the exception of the final 5mμ residue, contained the same sugars in similar proportions as the initial starch-free polysaccharide. The final residue contained only glucose, and is thought to be the starch type glucan described in earlier work.[50] Again no fractionation based on composition was obtained.

While the results of these studies are not direct chemical proof of the heteropolymeric nature of the extract, they do represent strong indicative evidence. Certainly as a result of this work much more weight must be given to the theory that polysaccharide and protein are strongly

associated. For more absolute chemical evidence for the existence of this extract as a single polymeric species the techniques of:-

(a) Smith's Degradation,

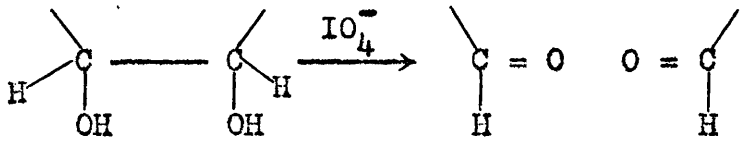
(b) Partial acid fragmentation,

(c) Desulphation and Methylation,

were investigated.

SMITH'S DEGRADATION

Periodate oxidation of a polysaccharide has for many years been used to obtain structural information. Wherever 1-2 diol systems occur in constituent sugar residues oxidation with sodium metaperiodate causes cleavage of the carbon-carbon bond with resultant conversion to the dialdehyde i.e.

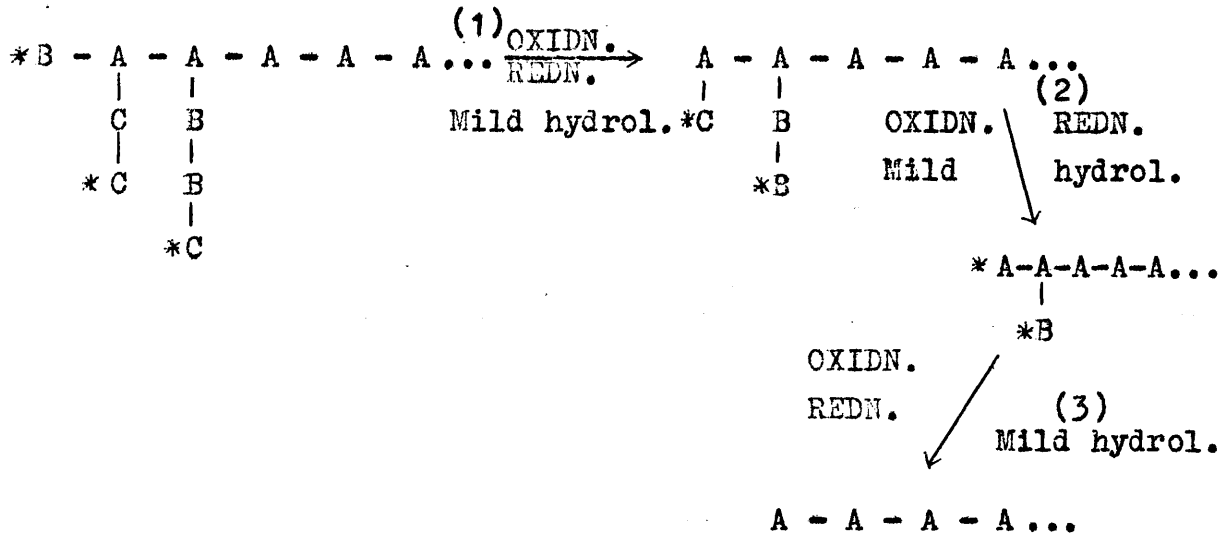


Hydrolysis of the polyaldehyde (oxidised polysaccharide) gives cleavage products diagnostic of the type of units and linkages present in the polymer. This system, however, has certain drawbacks, firstly, some of the products are unstable in the acidic conditions used during hydrolysis, and,

secondly, intra- and inter-molecular acetal formation between free aldehydes and hydroxyls is likely to occur. To avoid these problems Smith and co-workers[103] converted the polyaldehyde into the reduced derivative, the polyalcohol.

At a later stage it was observed that there was a significant difference in the rate of hydrolysis of the acyclic and cyclic acetal (glycosidic) linkages, the latter being the more stable.[104] It is therefore possible to use sufficiently mild acidic conditions which will cause hydrolysis of the true acetal linkages only, leaving the glycosidic links intact. A single Smith degradation (sequential periodate oxidation reduction and mild acid hydrolysis) of a polysaccharide removes all the residues in the molecule which contain α-glycol groups and new vicinal hydroxyl groups are exposed in the residual polymer.

Partial hydrolysis of a molecule, in which many of the residues are not attacked by periodate, leads to the production of a high molecular weight degraded polymer at the end of the sequence. This material can then be given a series of such degradations which gradually erode the molecule down to a core. For example



* = MOLECULE SUSCEPTIBLE TO CLEAVAGE

The system above indicates, primarily, that it is possible under the correct circumstances, in particular conditions which avoid over-oxidation, for all the A core to be revealed as long as "in-chain" A residues are not attacked by periodate. A second feature is that the number of units cleaved in the primary oxidation is always equal to, or greater than, the number of units cleaved in the subsequent oxidation, since, to obtain secondary oxidation it is necessary for previously cleaved units to be removed setting free suitable hydroxyl groups for subsequent oxidation.

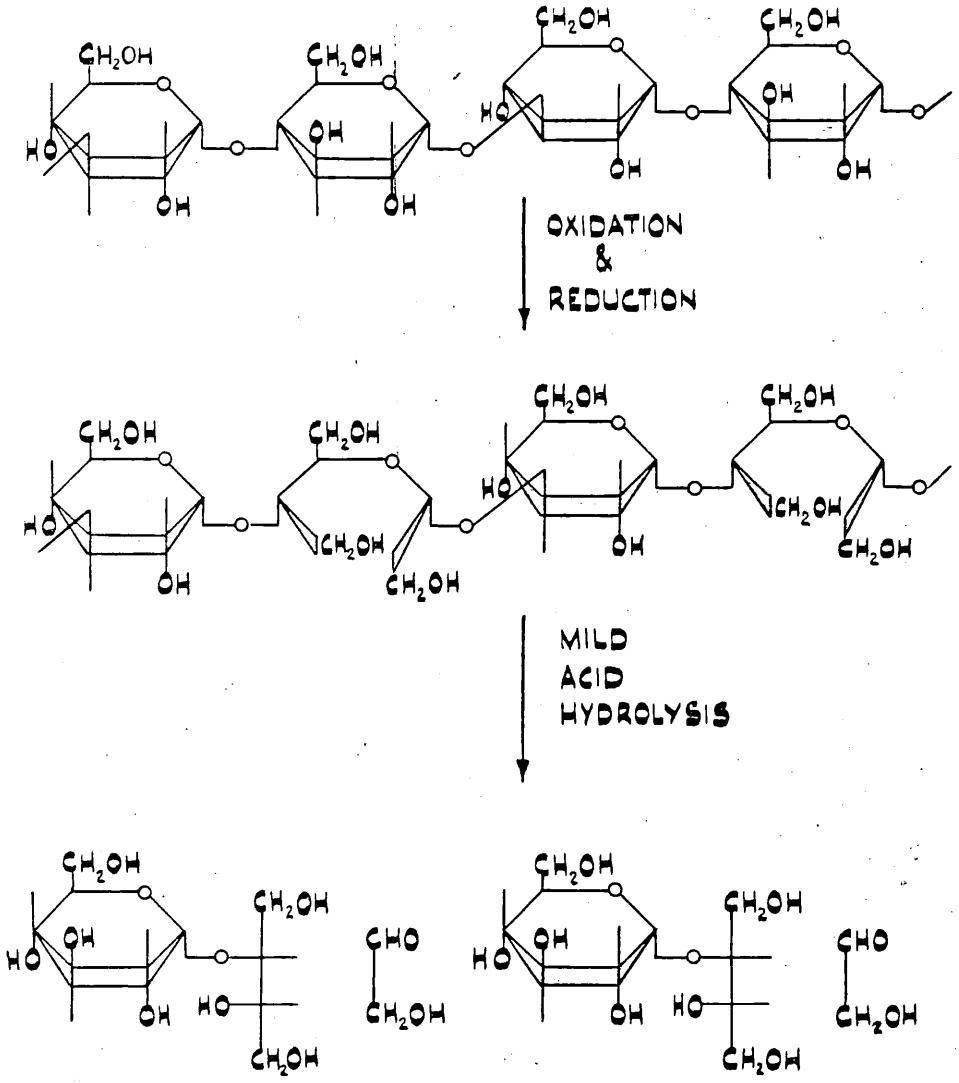
Furthermore, the fragments obtained from the mild acid hydrolysis (as distinct from the complete acid hydrolysis)

represent components which may be indicative of the type of unit and of the nature and order of linkages. An example will illustrate these points. Nigeran is a linear polymer of α,1,3- and 1,4-linked D-glucopyranosyl residues. The arrangement of these linkages in the macromolecule can be shown from the fragments isolated from Smith's degradation studies (Figure 8).

The fact that the only fragment containing uncleaved sugar is glucosylerythritol (2-O-α-D glucopyranosyl-D erythritol) in a system known to contain only 1,3 and 1,4 links is proof of an alternate 1,3 linked (periodate non-vulnerable), 1,4 linked (diagnosed from the presence of erythritol) system.

This procedure for degrading polysaccharides has been applied to the hot water-soluble extract from C. rupestris in an attempt to obtain evidence in favour of the hetero-polymeric nature of the extract, and to deduce some aspects of its internal structure, both from the gross degradation and from the fragments obtained.

Initial studies on the conditions and methods to be used for the degradation indicated that the destruction of excess periodate was best carried out with ethane 1-2 diol,



2-O-α D-GLUCOSYL ·D· ERYTHRITOL

ETHANE 1-AL 2-OL

FIGURE 8

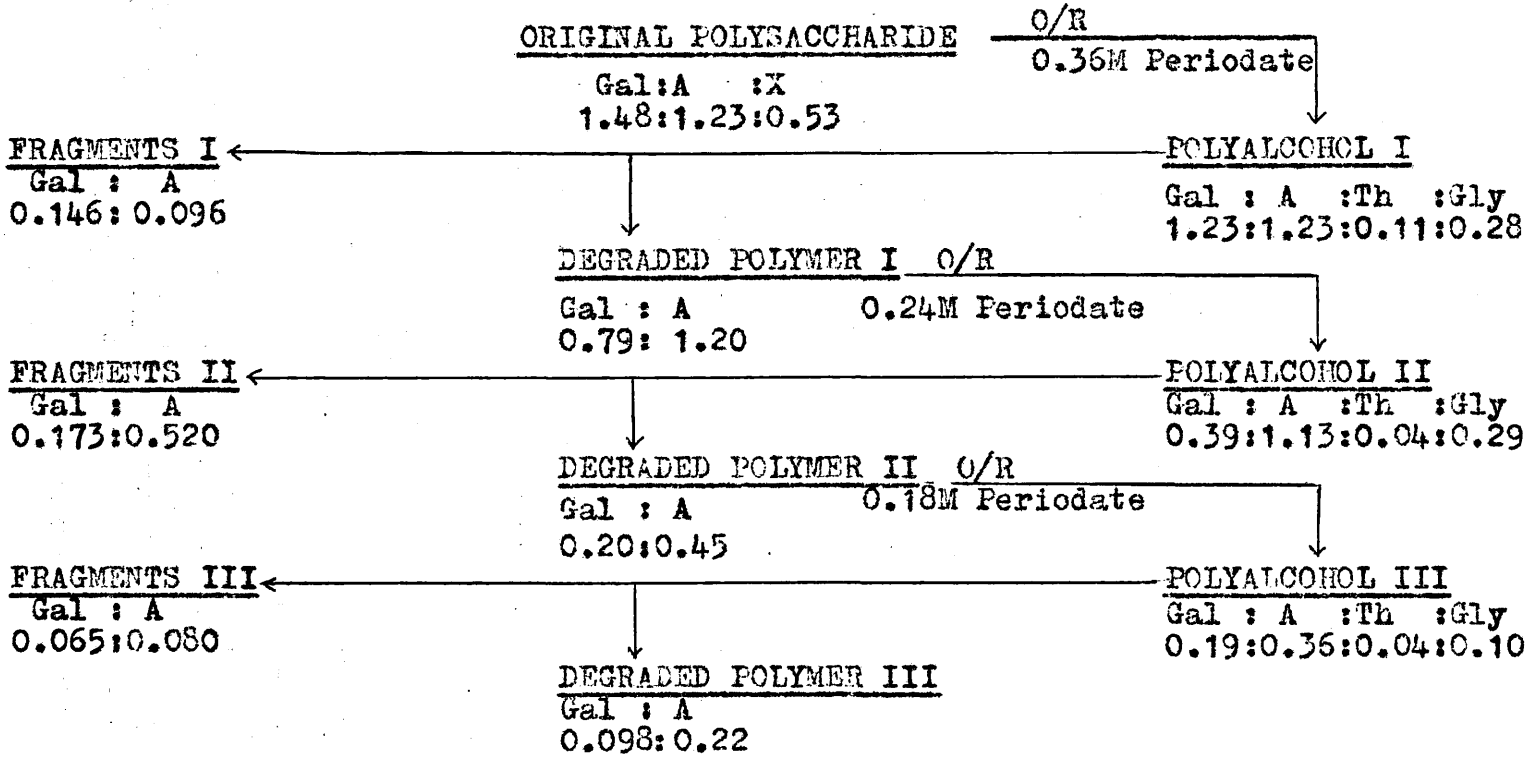
attempts to halt oxidation using sulphur dioxide failed to give satisfactory results since the solution was liable to become contaminated with colloidal sulphur. To avoid excessive alkalinity during the reduction phase it was found that the use of 0.05M-boric acid buffer maintained the pH between 9 and 10. The use of carbon dioxide gas in this context was equally successful but the ancillary equipment required to maintain an even flow of gas through the solution made the procedure more cumbersome.

It was found, in practice, that the polyaldehyde tended to form an insoluble product after alcohol precipitation, this was avoided by dialysing the oxidation mixture, and then, reducing without isolation of the polyaldehyde. It was later demonstrated that no loss in yield occurred even if reduction was carried out directly after oxidation, without prior dialysis. As a result the polyaldehyde solution, after oxidation, was treated with borohydride and, after the relevant period elapsed, dialysed. Since the materials were examined for alcoholic fragments great care was taken to avoid contamination with glycerol from the dialysis tubing, to this end, the polyalcohol after dialysis was always precipitated from solution with alcohol before freeze-drying.

The process of mild acid hydrolysis was initially performed using 0.1N sulphuric acid for 18-24 hours at room temperature. This, however, necessitated the use of barium carbonate to neutralize the alcoholic acid solution containing the fragments. As a result of this, the latter became badly contaminated with any soluble excess barium carbonate, which made chromatography very difficult. A cleaner technique was devised, this used the autohydrolysis procedure (see p.156). That hydrolysis of only the acetal linkages occurred during this process, was demonstrated by paper chromatographic examination of the fragments. No free sugar could be detected. Furthermore, proof of the complete cleavage of this type of linkage was derived from the fact that examination of a complete hydrolysate of the recovered degraded polymer showed the absence of any fragments from the oxidised sugars, only uncleaved monosaccharides being obtained.

The polysaccharide was subjected to three sequential degradations, the results of which are given in the following flow diagram (Figure 9).

FLOW DIAGRAM OF SEQUENTIAL SMITH'S DEGRADATION



Periodate reduction in Moles/Anhydrohexose unit

X = Xylose
A = Arabinose
Gal = Galactose
O/R = Oxidation & Reduction

All figures are quoted in gram quantities.

FIGURE 9

It was considered that a clearer picture of the degree, and extent, of oxidation of this complex polysaccharide would be obtained if all the products were expressed as respective weights of their constituents. The weights of the individual sugars were calculated from the total carbohydrate content, and their relative proportion figures obtained from the peak areas of their TMS derivatives on g.l.c. Since the relative proportions of the alcoholic derivatives, to those of sugars were known, using the same system, their weights could also be calculated.

By g.l.c., the peaks of all components were readily separable and only a minimum overlap occurred between the second xylose peak and the first galactose peak (see page 74).

It can be seen from the flow sheet that the first oxidation proceeded in a manner analogous to previous studies,[56,59] the loss of xylose being total and a 15% loss of galactose occurring (a total loss of 800 mg). There appears to be no loss in arabinose although earlier methylation studies[56] had indicated the presence of a small proportion of 1,4-linked, and therefore, periodate vulnerable, arabinose units. However, these could well have been residues in the initial polysaccharide carrying ester sulphate at C-3 which lost their sulphate under the highly alkaline conditions of methylation.[39]

The low molecular weight alcohols, glycerol and threitol, obtained on complete hydrolysis of the polyalcohol I are of interest, both in the qualitative and quantitative sense. Glycerol is to be expected from a 1,4-(1) or 1,5-(2) linked pentose, or a 1,6-(3) linked galactose residue (see Figure 10).

Similarly, threitol can be derived from galactose both 1,4 linked pyranose (4) and 1,5/6 linked furanose (5) (see Figure 11). Thus the two fragments in themselves are not absolutely diagnostic, except that threitol can only be derived from galactose of an uncertain ring type.

The presence of D-threitol in polyalcohol I has been established. Since rhamnose and threitol have similar chromatographic mobilities preparative separation on Whatman No.17 paper gave a mixture of the two components. After oxidation, with bromine, of the rhamnose to rhamnonic acid, they were separated on an Amberlite IRA 400 (OH^-) column. The crystalline D-threitol was recovered from the aqueous eluate and was further characterised as the crystalline 1,3-monobenzylidene derivative.[86]

The calculated quantity of galactose required to produce the amount of threitol, measured experimentally, corresponded to the majority of the galactose lost in the oxidation (also experimentally determined). Similarly, the xylose cleaved

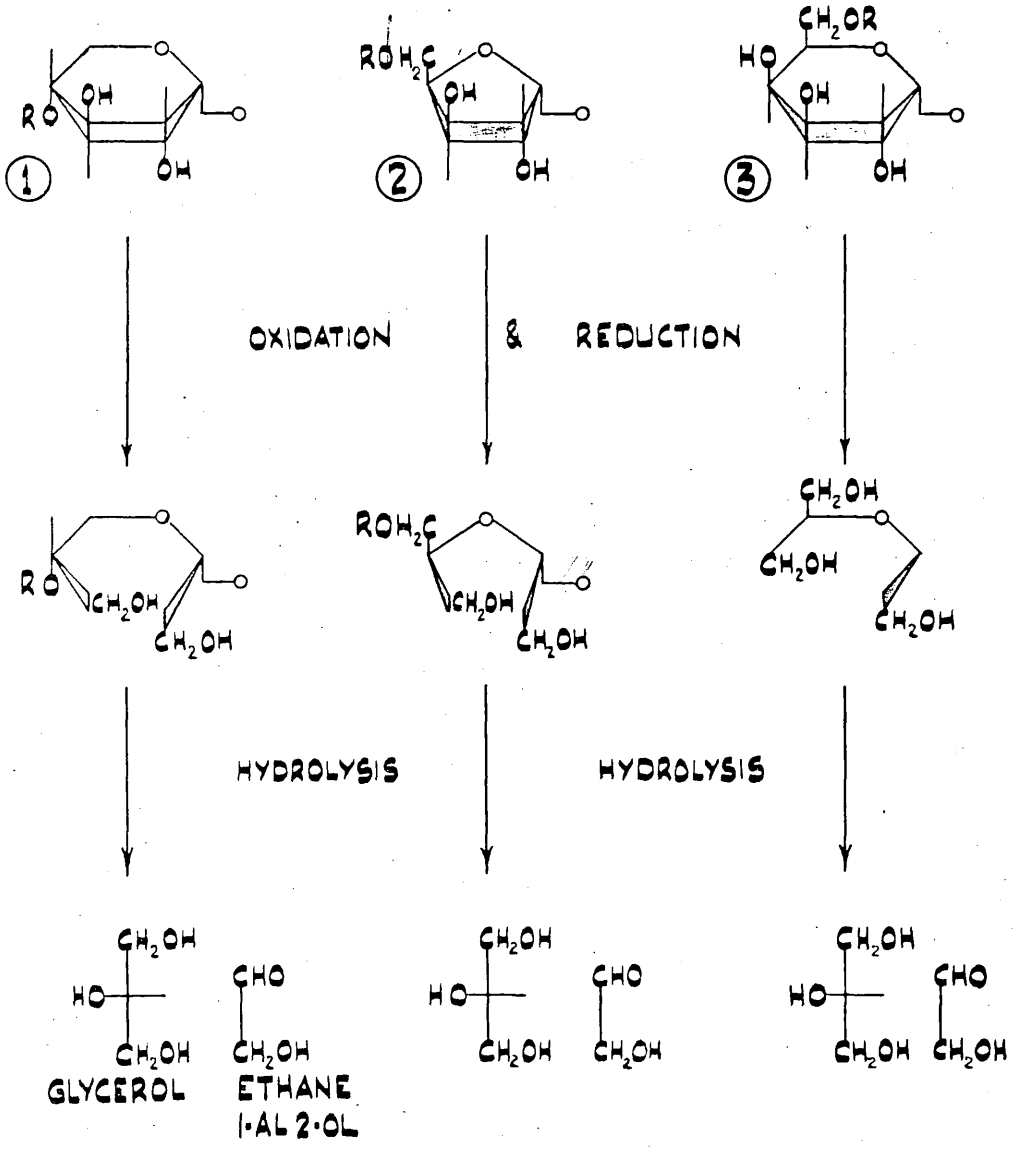

RO
OH
OH
1
ROH2C
OH
OH
2
CH2OR
HO
OH
OH
3
OXIDATION
&
REDUCTION
RO
CH2OH
CH2OH
ROH2C
CH2OH
CH2OH
CH2OH
CH2OH
CH2OH
HYDROLYSIS
HYDROLYSIS
CH2OH
HO
CH2OH
GLYCEROL
CHO
CH2OH
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FIGURE 10

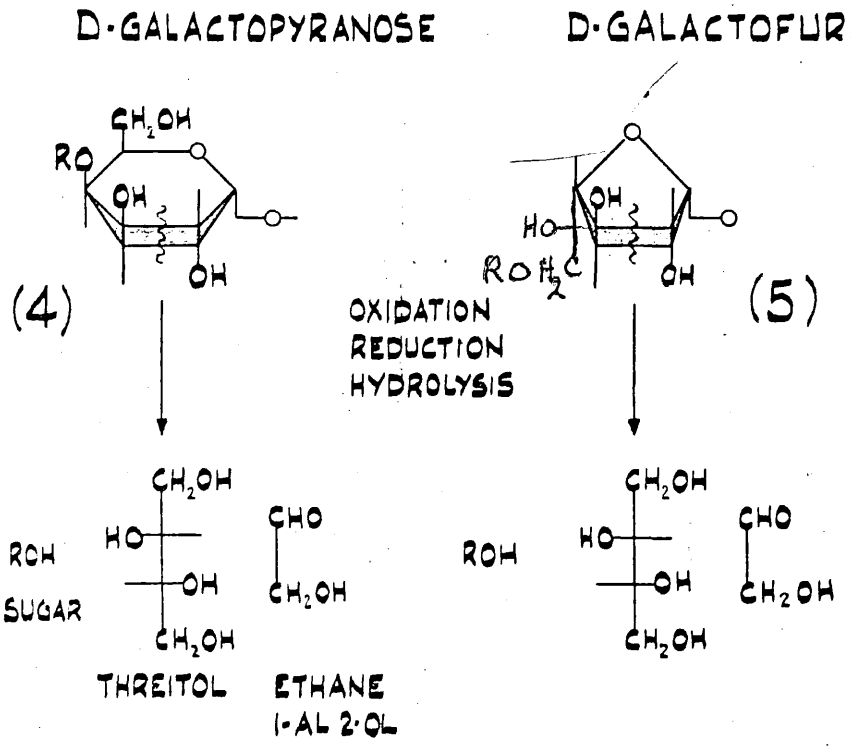


FIGURE 11

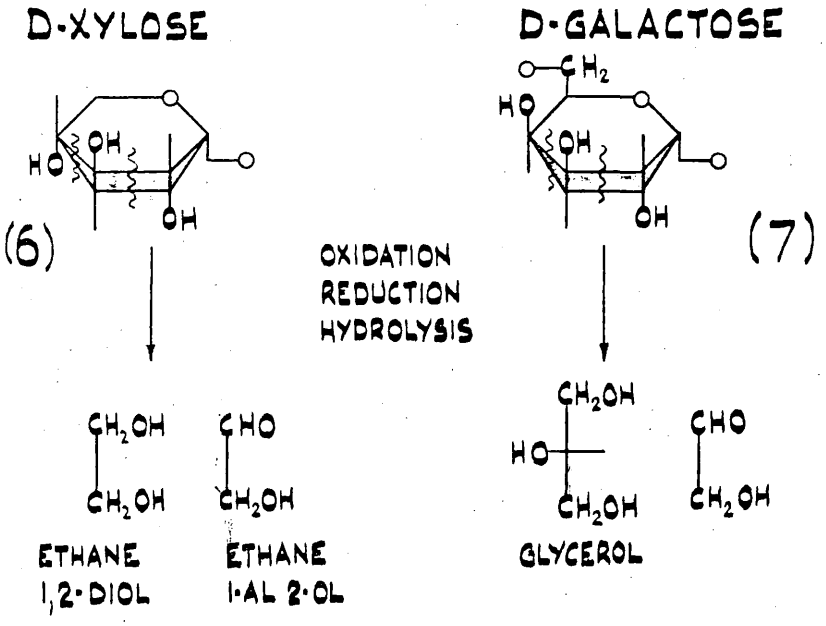


FIGURE 12

on oxidation accounts for a high proportion of the glycerol produced, the difference in the figures being a result of end-group xylose converted to ethane 1-2 diol (6). This difference cannot be quantified to give the proportion of end-group xylose in the macromolecule since end-group galactose gives rise to glycerol (see Figure 12).

The fragments from the partial hydrolysis (1st Degradation) were separated by preparative ionophoresis into neutral and acidic components. The neutral components were glycerol (I_A), threitol (I_B), arabinosyl-glycerol (II), arabinosyl-threitol (III), galactosyl-glycerol mixed with arabinosyl-threitol (IV) and a component containing galactose, arabinose, glycerol and threitol, in approximately molar proportions, to which no absolute structure can be assigned (V).

The acidic components (I and II) were apparently mixtures and it can only be tentatively suggested that one possibility for the components (I) is an arabinosyl-diglycerol system mixed with galactosyl-threitol.

The presence of free glycerol and threitol indicates the existence of xylose adjacent to xylose and/or galactose and galactose adjacent to galactose and/or xylose units in the macromolecule, but it is not possible from these results to say how long these sequences are.

The presence of arabinose in these fragments shows that, although this sugar was immune to periodate attack, it was linked to units vulnerable to periodate (galactose or xylose) and this, and the presence of arabinosyl threitol, is the first positive proof that arabinose is a member of the heteropolymer.

The degraded polymers have been examined by Sagavac gel column chromatography. This cross-linked agarose matrix will allow molecules (proteins) of molecular weight 1×10^7 to permeate the gel. It can be shown that, for polymers of similar shape, a relationship exists between the elution position from the column with the molecular weight of the component. Thus a particular column can be calibrated for proteins using standard proteins of known molecular weight, or for carbohydrates using the Sephadex dextran series, where each of the dextrans has a very small molecular size dispersity and hence can be given a single value for its molecular weight. This system is very sensitive to molecular shape, and hence calibration must be carried out using suitable standards. Since, at this time, there are no suitable standards for highly branched sulphated polymers, no attempt was made to calibrate the column, but the makers figures of a fractionating range of 1.0×10^7 to 1×10^4 were

used to obtain relative values for the molecular weights of the different samples and a rough idea of their absolute magnitude.

The void volume and exclusion volume of the column were determined using lyophilised E. coli cells. This is a mixture of cell debris, which elutes at the void volume, and low molecular weight proteins, which elute at the exclusion volume. The assumed molecular weight was calculated using the following formula.

$$\text{Log}_{10}\text{Mol.Wt.} = F(\log_{10} 10^3) + \log_{10} 10^4$$

$$\text{where } F = \left(\frac{\text{exclusion vol} - \text{elution vol}}{\text{exclusion vol} - \text{void vol}}\right)$$

The degraded polymer I had a maximum carbohydrate content at a volume equivalent to a molecular weight of about 5% of that of the original polymer (see Figure 5) [p77]. Assuming that cleavage in the interior of the molecule halves the molecular weight, it can be calculated that about 19 random cleavages in the interior would cause this reduction in molecular size. Since this represents only a small proportion of the cleavages, it follows that considerable degradation must also have taken place at the periphery of the molecule. Similar examination of the polyalcohol did imply some fragmentation of the molecule at this stage, but the basic elution pattern was similar to that of the polysaccharide.

The second oxidation caused the cleavage of 50% of the remaining galactose and about 7% of the arabinose (a total of 470 mg.). This is in accordance with the expected amounts relative to the first oxidation.

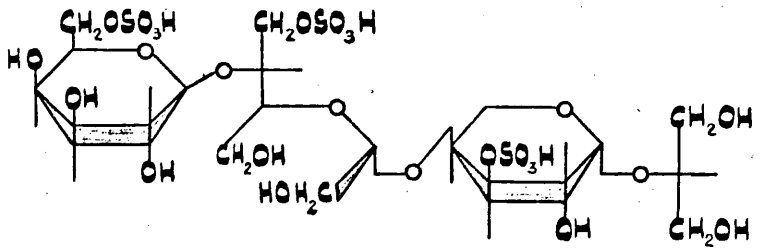
It is significant that a larger quantity of galactose was cleaved in the second oxidation compared with the first (400 mg. compared to 250 mg.). It follows that some of the galactose vulnerable to periodate in the second oxidation owed its immunity in the first oxidation to the presence of primarily vulnerable xylose, proof that these two sugars are mutually linked. Similarly cleavage of arabinose (70 mg.) in the second oxidation could only have occurred as the result of loss of attached galactose, and/or xylose, in the first oxidation, once more proof of mutual linkage. These results provide more positive evidence of the heteropolymeric and highly branched nature of the polysaccharide.

Mild acid hydrolysis of polyalcohol II caused extensive fragmentation in the molecule. The molecular weight distribution of the derived degraded polymer obtained by Sagavac gel chromatography gave a plateau shaped curve (p.77) with a widespread maxima, the average of which was 4.7% of that of the original polysaccharide. Furthermore 75% of the total

carbohydrate found in the fragments was represented by a non-reducing acidic oligomeric mixture of not less than 4 units in size (chromatographic mobility) comprising arabinose and galactose in the ratio 3:1. The only pure fragments of a certain structure were threitol (I) and arabinosyl-glycerol (II), the rest represented mixtures of uncertain structure.

The reason for the extreme fragmentation is not clear since, either the galactose or arabinose could have been responsible. The significance of this observation will be discussed at a later stage in connection with other factors (see p.182).

The final oxidation caused cleavage of 5% of the remaining galactose and 20% of the remaining arabinose (90 mg.). In the second oxidation only 70 mg. of arabinose was lost and the additional loss in the final degradation must be the result of removal of galactose during the second oxidation, evidence once more of mutual linkage. The fragments obtained by mild acid hydrolysis were free threitol (I), arabinosyl glycerol (II), and two sulphated species (III), and (IV). The component (IV) is believed to be a single material containing all four components, galactose, arabinose, threitol, and glycerol in molar proportion. It would be expected to have a structure based on the following system (component 1), the order of which is not absolute but which is stable as a result of the shielding effect of the sulphate ester group.



COMPONENT 1

The material has a high M_{GA} value but is slow in the basic solvent used in analysis. Whether or not this is the correct structure it is believed to represent evidence in favour of the mutual linkage of galactose and arabinose.

The molecular distribution of the final degraded polymer was much more like that of degraded polymer I. The degradation procedure would seem to have caused more damage to molecules in the upper molecular weight range. The molecular weight is reduced to a value of 4.1% of the original polymer.

The water soluble degraded polymer from the final degradation (D.P. III, 40% carbohydrate, 36% sulphate) was examined by DEAE-cellulose column chromatography in an

attempt to see whether homopolymeric fragments might now be separated and also to see whether a low sulphate content fraction could be obtained, which might be suitable for methylation.

The polymer did fractionate using this system but all fractions contained the same sugars in the same proportions but with varying sulphate content. Unfortunately much of the material remained irreversibly held on the column matrix which even alkaline elution failed to remove. No fraction was obtained with a sulphate content low enough to allow meaningful methylation (p 87-88). However the results of the fractionation imply that the fragmented polysaccharide is still heteropolymeric.

Some of the results obtained during this procedure will be discussed later in conjunction with results from the remaining analytical procedures.

Some of this work has been published and the paper is included at the back of this thesis.

FRAGMENTATION STUDIES

The fragmentation studies undertaken have been autohydrolysis and acetolysis.

Autohydrolysis was developed from work by Painter,[105] in which he used a polystyrene sulphonic acid to hydrolyse a polysaccharide. Both acid and carbohydrate were polymeric and were retained inside dialysis tubing during the hydrolysis reaction. The products of hydrolysis were, however, capable of passing through the membrane and hence could escape into the neutral solution (distilled water) surrounding the sac. This means that as a fragment forms in the well-stirred system, it passes out of the acidic environment almost immediately and thus suffers a minimum of further hydrolysis. In this way a high percentage of oligomers results from this treatment.

The extension which autohydrolysis has made to the method is that the polysaccharide becomes its own hydrolysing agent. When an acidic polysaccharide (one containing sulphate ester groups for example) is treated with a cationic resin (e.g. Amberlite IR 120(H^+)) the free acid form of the polysaccharide is generated. If this is then heated inside a dialysis sac hydrolysis occurs and the fragments pass out into the solution surrounding the sac. As a result a large

number of acidic fragments are produced; in this instance the surrounding solution is no longer neutral but represents a dilution system which at low concentration (50 μ g/ml) also minimises further hydrolysis.

The use of autohydrolysis has been reported only once when it was used to partially hydrolyse a glucuronoxylofucan from Ascophyllum nodosum.[62] In this case a very high proportion of sulphated oligomers were produced (ca. 60%).

This method of partial acid hydrolysis has been applied in two different ways to the hot water soluble extract from C. rupestris.

Firstly, the free acid form of the polysaccharide was heated in a closed dialysis sac (EXPT.17) for three periods of two hours each. From the dialysis sac insoluble material (ca. 22.5%, with a high nitrogen content) and a soluble component (43%) were recovered. The latter containing proportionately more arabinose than the initial polysaccharide and considerably less galactose (about equal to the amount of xylose). Rhamnose, although still a minor constituent, had increased somewhat. These results follow the expected pattern of partial hydrolysis of this extract. A large proportion of the galactose is very acid labile and both it, and some of the xylose, appear to be on the periphery of the

molecule and consequently more readily hydrolysed.

All four monosaccharides were present in the dialysate together with a number of neutral and acidic oligomers. This material had a low carbohydrate content and was purified on an anionic resin column, during which the acidic fragments were discarded. The neutral oligomeric components were subsequently separated from the monosaccharide fraction using a charcoal-Celite column, the former being eluted with 5% ethanol and the latter with water.

The first oligomeric fraction, which gave a single spot on a paper chromatogram in several solvents, had a DP of 2 and gave galactose and xylose on hydrolysis. This was considered to be a mixture of 4-O-D-xylopyranosyl-D-xylopyranose and 4-O-D-galactofuranosyl-D-xylopyranose, since after reduction and hydrolysis the only alcohol detected was xylitol, and after methylation and methanolysis methyl 2,3,4-tri-O-methylxyloside, 2,3-di-O-methylxyloside and 2,3,5,6-tetra-O-methylgalactoside were the only sugars which could be detected. It is to be expected that disaccharides of this type would have very similar chromatographic mobilities.

The second component also comprised a mixture of disaccharides which gave a single spot on chromatographic

analysis. In this instance however galactitol was the only alcohol found after reduction and hydrolysis. Methylation studies were consistent with the presence of 3-O-D-xylopyranosyl-D-galactose and 3-O-D-galactofuranosyl-D-galactose. Again the chromatographic mobilities and properties were in agreement with these structures.

The third oligomer had the chromatographic mobilities and properties of 6-O-β-D-galactopyranosyl-D-galactose and this was confirmed by methylation. This disaccharide had previously been isolated and characterised by Mackie and Percival from a hydrolysate of this polysaccharide.[55]

In the second method (EXPT. 19) autohydrolysis was allowed to proceed until all the polymeric material had been fragmented. Dialysates were removed when the carbohydrate content approached 50μg/ml. Chromatographic analysis revealed the essential similarity of the different dialysates which were therefore combined and fractionated on No.17 paper into monosaccharides and four oligomeric materials. The first of these (FRACTION IV) was separated by preparative ionophoresis into galactose-6-sulphate, 1,4 arabinobiose 3-sulphate and 1,3-galactobiose, materials separated in earlier hydrolytic studies of this polysaccharide.[55]

The second fraction (FRACTION V EXPT 22) was chromatographically and ionophoretically homogeneous and again had a DP of 2, but the fact that it contained galactose and arabinose in the molar rates 1:1.6 indicated that it was a mixture of disaccharides. This was supported by the presence of both galactitol and arabinitol in a hydrolysate of the reduced material. The sulphate content corresponded to a molar ratio of 0.83:1.0 for sulphate to mixed anhydrohexose/pentose, indicating one sulphate per sugar unit which agrees with its M_{GA} value of 1.2. It appears that the sulphate content governs the chromatographic mobility since, from the molar ratios, in addition to mixed disaccharides, there must be some arabinobiose disulphates in this fraction.

Methylation and analysis of the methyl glycosides of the derived methylated sugars supported the presence of a number of different disaccharides.

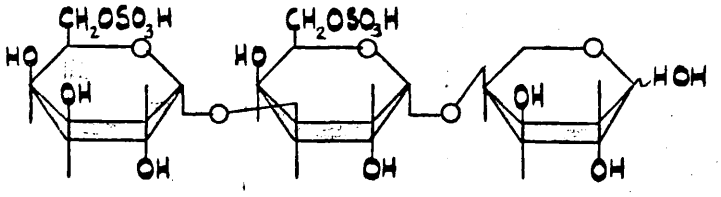
After reduction and removal of alkali labile sulphate, it proved possible to separate on ionophoresis a neutral and an acidic material. Both these on hydrolysis gave galactose, arabinose, xylose, and galactitol. The acidic material also contained arabinitol, which could be considered evidence for an arabinobiose in this mixture.

Xylose is the expected product from the desulphation of arabinose 2 or 3 sulphate (see p.30).

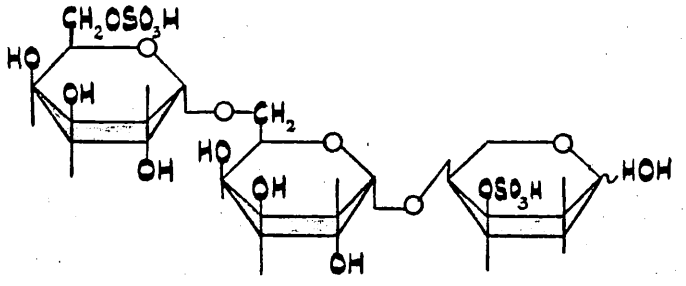
These results lend further support for the presence of a sulphated arabinosylgalactose. The presence of the monosaccharide galactose in the neutral desulphated materials is more difficult to understand since, no tetramethyl galactose could be detected in the g.l.c. of the methylated highly sulphated fraction V. However, since this oligomer is only a small part of the total mixture and, also, since the region where the galactose derivative is found on the chromatograph is crowded with other peaks, it would not be difficult to fail to distinguish a small peak diagnostic of tetramethyl galactose.

The third fraction (VI); (p100) was an acidic trisaccharide containing arabinose and galactose in the molar ratio of 1:2. The sulphate content was approximately equal to 2 moles of sulphate for every trisaccharide unit and partial hydrolysis yielded galactose-6-sulphate and arabinose-3-sulphate. That arabinose was the reducing unit, was shown by the presence of arabinitol as the only alcohol in the hydrolysate of the reduced trisaccharide. Methylation results indicated the presence of galactose substituted at the 6 position and also

disubstituted at the 3 and 6 position and arabinose substituted at the 4 and also disubstituted at the 3 and 4 position. A mixture of the two trisaccharides (2 and 3) would agree with all these results.



COMPONENT 2



COMPONENT 3

The formation of 3,6-anhydrogalactose on alkaline treatment of the trisaccharide, confirmed the presence of 6-sulphated galactose units in which the hydroxyl group on C-3 is unsubstituted .

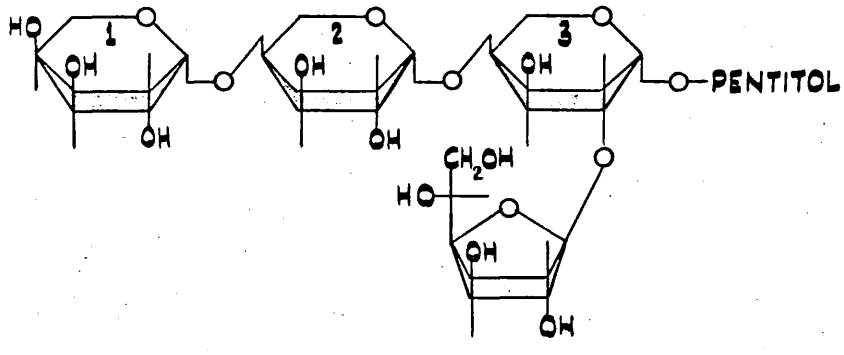
It was hoped to determine the proportion of the two trisaccharides by oxidation of the reduced material with

periodate under conditions which would only oxidise the arabinitol (G.M. 16). Reduced trisaccharide (2) should only react with two moles of periodate and reduced trisaccharide (3) with one mole, both should liberate one mole of formaldehyde. In practice it was found that 2.2 moles of periodate were reduced and 0.75 moles of formaldehyde released for every trisaccharide unit. The presence of sulphate on C-3 of trisaccharide (3) may well inhibit the oxidation and hence, explain the low release of formaldehyde in the present experiment. In contrast the slightly larger reduction of periodate could be an indication that other units in the trisaccharide are being oxidised . The most that can be concluded from the oxidation experiments is that in all probability the trisaccharide 2 is the major component.

The final fraction, VII, which constitutes 66% of the arabinose, proved to be a mixture of sulphated pentasaccharides in which the molar ratio arabinose to galactose is 4:1. Partial hydrolysis gave, in addition to the free sugars, arabinose-3- and galactose-6-sulphate, as well as a number of oligomers, none of which had the mobility of the earlier fragments.

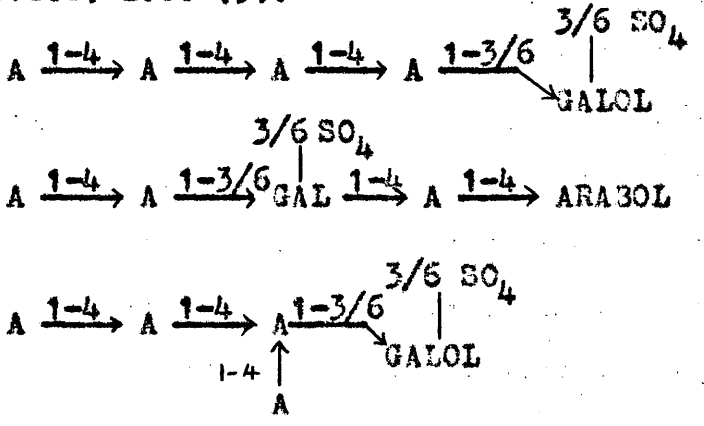
Arabinitol, and a trace of galactitol, were detected in a hydrolysate of the reduced material. Methylation studies

showed that 3,4-linked arabinose was the major constituent and that 2,4- and 4-linked arabinose were also present. The major galactose unit was the 3,6-linked specie but a small amount of end group galactofuranose was also indicated. Alkaline desulphation failed to produce any 3,6-anhydrogalactose but from the desulphation mixture it was possible to separate a neutral fraction. Methylation of this and analysis of the derived methylated glycosides, showed that it comprised end group xylose (trace) and arabinose, 4-linked arabinose and xylose, 2,4-linked arabinose and end-group galactofuranose, the xylose being derived from 3-sulphated arabinose. The neutral oligosaccharide, therefore, has the constitution (4) (units 2 and 3 can be reversed).



COMPONENT 4

The original pentasaccharide, from which this was derived, was undoubtedly mainly sulphated on units (3) and (2) probably at C-3. The residual acid material (comprising about half the original fraction) gave the same arabinose and xylose derivatives on methylation as the neutral oligosaccharide, but the only methylated galactose which could be detected was the 2,4-di-O-methylsugar. This implies, firstly, that the acidic component is a mixture of species both linear and branched, and secondly, that the galactose mainly exists linked, or sulphated, at C-3 and C-6. It may be in an inter residue position or at the reducing end group (hence the trace of galactitol in the hydrolysate of the reduced species) i.e. (5).



A = ARABINOSE
GAL = GALACTOSE
ARABOL = ARABINITOL
GALOL = GALACTITOL

5

The presence of end group xylose, derived from end-group arabinose sulphated at C-3 in these reduced desulphated

pentasaccharides, requires 2,4-di-O-methylarabinose as a constituent of methylated VII. This sugar has not been detected although the major sugar was 2-O-methylarabinose. It can only be concluded that the presence of ester sulphate on C-3 inhibits methylation of the adjacent axial hydroxyl group at C-4 in L-arabinose in the C1 conformation. This is most certainly true in the case of tosylation of D-mannose where the C1 form can be specifically tosylated at equatorial C-3 rather than at the axial C-2 even though the C-2 is normally expected to be more reactive.[106]

The sulphated form of the reduced pentasaccharide (4) should reduce six moles of periodate, whereas the reduced pentasaccharide (5), in the sulphated form, will react with 3-4 moles of periodate (on the basis of one vulnerable interchain residue and the influence of a trace of galactose at the reducing end group). The two components are present in approximately equal quantities and therefore one expects that the average molar reduction of periodate per pentasaccharide unit should be between 4.5-4.75 moles, which is in reasonable agreement with found value (4.9 mole). The increased proportion of uncleaved galactose to arabinose in the derived polyalcohol is supporting evidence that a large proportion of the galactose was 1,3,6-substituted.

The presumed monosaccharide material from the autohydrolysis was shown to be free from fast moving oligosaccharides by fractionation on Sephadex G.10 column (EXPT. 25) from which the xylose and galactose were recovered, reseparated, and estimated.

Although considerable losses were incurred during the purification of the individual fractions it can be seen from TABLE XXV that the overall relative proportions of the recovered sugars is in reasonable agreement with their proportions in the initial polysaccharide. Furthermore about 66% of the recovered arabinose is to be found in fraction VII.

FRACTION	CARBOHYDRATE		RELATIVE MOLAR PROPORTION OF MONOSACCHARIDE
	WEIGHT	% OF MIXTURE	
II	8 mg.	6.6%	XYLOSE 1
III	26 mg.	21.4%	GALACTOSE 1
IV	16 mg.	13.2%	GALACTOSE:ARABINOSE 3:1.2
V	11 mg.	9.1%	GALACTOSE:ARABINOSE 1:1.6
VI	20 mg.	16.5%	GALACTOSE:ARABINOSE 2:1.0
VII	40 mg.	33.2%	GALACTOSE:ARABINOSE 1:4.0
	121 mg.		

OVERALL RELATIVE PROPORTION

GAL	: ARAB	: XYL
1	: 0.76	: 0.12

TABLE XXV

Paper chromatographic examination of aliquots removed from each of the dialysates invariably showed galactose, galactose-6-sulphate, and xylose as the major monosaccharides with only traces of arabinose and rhamnose (see p.94). The oligosaccharides V, VI, and VII, were always present, but reached a maximum in the first dialysate and then gradually decreased until the final aliquots contained only traces of these materials. The absence of free arabinose can be explained as either, due to the relative stability of the arabinose, and/or to the fact that the majority of this sugar is situated in the inner part of the highly branched molecule; that its linkages are comparatively stable is shown by the high proportion of arabinose in the pentasaccharide fraction. All the evidence indicates that the xylose and a considerable proportion of the galactose units occur at the periphery of the macromolecule and this, associated with the fact that much of the galactose is present in the furanose form, could explain the lability of these units to acid hydrolysis.

These hydrolytic studies have provided conclusive proof of the heteropolymeric nature of the extract, of the relative acid lability of the xylose and much of the galactose, and of a major structural unit comprising four 1,4 linked arabinose

units associated with a single galactose. It appears that no single sugar constitutes a backbone for the molecule, but that the inner part is made up of a repeating unit based on the pentasaccharide, linked together in an uncertain manner. The trisaccharide (VI) may well constitute a side branch or a component of the main chain.

The variety of disaccharides is so complex that little can be said, except that the arabinosylgalactose may well have been derived from the periphery of the molecule. It should be remembered that arabinosylothreitol was recovered from the fragments in the first Smith's degradation, supporting the presence of such a disaccharide.

Attempts to prepare a degraded polymer with a low sulphate content from autohydrolysis (see p.106) failed. In one experiment the hydrolysis was stopped when the pH of the mixture rose and the residual polymer [a soluble (65% carbohydrate) and insoluble (6% carbohydrate content) component] in the dialysis sac recovered. The soluble material was found to have a higher sulphate content than the initial polysaccharide. Attempted fractionation of the water-soluble material on DEAE-cellulose was unsuccessful. It contained all the original sugars and gave a long streak

on ionophoresis. Analysis on a Sephadex G.50 column gave a broad peak with an estimated maximum DP of 16-22 units (p95). The insoluble component contained equal quantities of arabinose and galactose.

ACETOLYSIS

Acetolysis of the desulphated polymer (see EXPT. 27) gave very poor yields of oligosaccharides and it was only possible to separate μg. quantities which were tentatively characterised as a rhamnosyl (1 → 4) xylose (I); a mixture of rhamnosyl (1 → 4) rhamnose, and arabinosyl (1 → 4) arabinose (II); a rhamnosyl (1 → 3) rhamnosyl (1 → 3) rhamnose (contaminated with free galactose) (III) and (IV); and a trisaccharide containing two moles of rhamnose and one mole of galactose (V), the former occurring as end group and 1,3 linked units, and the galactose as a 1,6-linked unit. The 1,6-linked galactobiose (VI), a product of earlier studies was isolated from the acetolysis mixture.

This is in fact the first direct evidence that the rhamnose is a constituent of the heteropolymer but this can only be regarded as a preliminary study. It will be necessary to repeat the procedure on a larger scale and carry out a more rigid characterisation of the different oligosaccharides before this can be accepted without question.

DESULPHATION AND METHYLATION

The removal of sulphate ester groups from polysaccharides using methanolic hydrogen chloride was first reported in 1957,[107] when Kantor and Schubert noted the effect this reagent had on chondroitin sulphate during a study concerned with the reason for the lack of the metachromatic staining reaction of cartilage after treatment of this material with methanolic hydrogen chloride. Although desulphation by this reagent has been applied successfully to the polysaccharide extract from Ulva lactuca which contains uronic acid and ester sulphate groups, it seemed unlikely to be successful in the case of C. rupestris which is devoid of the stabilising uronic acid residues. Earlier studies[56] had[108] revealed the impossibility of completely methylating Cladophoran. Experiments were therefore instituted to prepare if possible a less highly sulphated polymer.

This reaction with methanolic hydrogen chloride, though never fully investigated, is supposed to occur via a transesterification procedure i.e.

$$M^+ \bar{O}SO_2\text{-}O\text{-}R + MeOH \xrightarrow{HCl} ROH + M^+ \bar{O}SO_2OMe$$

Acyl fission is presumed to take place, since alkyl oxygen fission would give isomers, a feature never before observed.

Initial studies with Cladophoran using 1% methanolic hydrogen chloride confirmed the earlier results of Mackie and Percival[55], in that, at no stage was a low sulphate polymer formed. By reducing the concentration of hydrogen chloride to 0.35% it was possible, after 48 hours treatment, to obtain a 45% overall yield of material in which the carbohydrate content had risen to 70% (representing a 63% overall yield carbohydrate) and the ester sulphate had been reduced to 10.4%. Continuing the treatment for a total of 96 hours resulted in a loss of material down to 17%, in which the carbohydrate content was only 33% with a sulphate content of 18% and arabinose as the major sugar, not the expected galactose. It seems that the removal of ester sulphate renders some of the glycosidic linkages more susceptible to methanolysis.

In view of these results a large scale hydrolysis with 0.35% methanolic hydrogen chloride was stopped after 48 hours. Analysis of the recovered polysaccharide (yield 50%; carbohydrate content 70%; sulphate 10.6%) showed, as was to be expected, a relative decrease in the proportion of galactose and xylose (see TABLE XXVI).

POLYSACCHARIDE (WT. OF CARBOHYDRATE)	GALAC-TOSE	ARABI-NOSE	XYLOSE	RHAM-NOSE
INITIAL - 100 mg	45.2mg	38.0mg	16.7mg	TRACE
DESULPHATED - 70 mg	20.59mg	38.3mg	9.06mg	TRACE

TABLE XXVI

Attempted methylation of this partially desulphated material using Srivastava's procedure resulted in a low (4%) methoxyl content probably because the polysaccharide gradually precipitated from the solution during the reaction. The recovered polysaccharide (70%) was subjected to four successive Haworth methylations (the most consistently successful method for sulphated polymers) and the product (ca. 100% yield) divided into a chloroform soluble material (A) (OMe found 21.3%) and chloroform insoluble material B (OMe 15.7%). The latter was further methylated by the procedure of Kuhn and the product, combined with the chloroform soluble component, subjected to methylation with Purdie reagents. The product (recovered in an overall yield of 30%) had a methoxyl content of 28.4%. An approximate value for the theoretical methoxyl is about 39% based on a polymer containing mainly di-O-methyl arabinose about 10% of tri-O-methyl hexose and 10% of sulphate ester. In order to raise the methoxyl content, fractionation of the methylated material with light petroleum/chloroform mixtures by the

method of Chanda et al.[109] was carried out. Five fractions were separated (see p.118) which had methoxyl contents (corrected) varying from 31% - 39% and sulphate contents from 10.6 - 2.3%. While four of the fractions had methoxyl and sulphate contents consistent with their solubilities in light petroleum, the first fraction, soluble in 80% light petroleum, had a methoxyl content of 33% and a sulphate content of 10.6%. It can only be concluded that this material was contaminated with other light petroleum-soluble materials and had in fact a higher methoxyl content.

For the separation and characterisation of the individual methyl sugars the three major fractions Q, R, and S, (methoxyl 35.7-39.7%; sulphate 2.3-5.6%) were hydrolysed since it had been shown (p.119) that all the fractions had an essentially similar content of methylated sugars. Fractionation of the hydrolysate on a cellulose column gave only a partial separation and fractions containing more than one component were reseparated on Whatman No.3 paper. The use of double and treble development of the papers was of the greatest value in the separation of materials with similar mobilities. Although the method has been used extensively in cellulose thin layer chromatography of monosaccharides[110] to give a longer effective running time, the method has only recently been used for paper chromatography of methylated derivatives

and as yet no report has appeared in the literature. Again, a longer effective running time is given since in many cases the solvent front was not allowed to run off the end of the paper. However, another probable cause of better separation is that during the second elution the fractionation between eluent and cellulose bound water is different from that of the first elution, since, the cellulose water shell will be expected to be partially saturated with the solvents present in the eluent. This means that the second elution does not result in fractionation between water and eluent with gradual saturation of the water, but in fractionation between water, partially saturated with eluent, and eluent. It should be noted that all papers were air dried for a maximum of only one hour.

Although the weights of all the fractions are recorded they were contaminated with material which was soluble in both alcohol and water and was not removed on treatment with charcoal. Nevertheless 2,3,4 tri-O-methyl-D-xylose, 2,3-di-O-methyl-L-arabinose, 2-O-methyl-L-arabinose, and 2,3,4-tri-O-methyl-D-galactose were characterised as crystalline materials or derivatives. This is the first time that the last three derivatives have been obtained as crystalline materials from methylated Cladophoran.

G.l.c. analysis of the glycosides derived from the hydrolysate of methylated fraction Q (OMe, 39.7%; SO_4^{2-} 5.6%) and calculation of the peak areas, gave the relative weights of the major sugars shown (TABLE XXVII). The methylated sugars, for which no weights are recorded, were present in very small proportions and totalled no more than approximately 7% of the total material. In each case their peaks were partially masked by the major constituents. The reason for choosing 135 mg. for the major constituent, 2,3-di-O-methyl-L-arabinose, as a standard for comparison arises from the fact that 135 mg. of this sugar were isolated in the preparative separation of the hydrolysate and this therefore, enables ready comparison of the two methods for determining the relative proportions of the different sugars.

The yields of the different methylated sugars obtained above and those recovered from the methylation of the initial polysaccharide are set out in TABLE XXVII.

The amounts of the major methylated sugars separated from the large scale hydrolysate are not entirely in agreement with the quantities calculated from the g.l.c. peak areas (see TABLE XXVII) although, apart from the 2-O-methyl arabinose, they are of the same order. The comparatively high recovery of the latter in the preparative separation is

METHYLATED DERIVATIVE	WEIGHT (FISHER[56])	WEIGHT (DESULPHATED)	WEIGHT g.l.c. DESULPHATED
2,3,4-TRI-O-METHYLXYLOSE	109.5	10.0	16.4
2,3,4,6-TETRA-O-METHYL GALACTOSE	45.4		-
2,3,5,6-TETRA-O-METHYL GALACTOSE	-		-
2,4-Di-O-METHYLRHAMNOSE	34.8	29.6	-
3,4-Di-O-METHYLRHAMNOSE			
2,3,5-TRI-O-METHYLGALACTOSE	152.8		-
2,3,4-TRI-O-METHYLARABINOSE	-		-
2,3-DI-O-METHYL-XYLOSE	85.5		-
2,3,4-TRI-O-METHYLGALACTOSE	205.8	16.5	14.3
2,3-Di-O-METHYLARABINOSE	135.0	135.0	135,0
4,O-METHYLRHAMNOSE	115.2	-	-
2,4-Di-O-METHYLARABINOSE	72.5	-	-
2,O-METHYLARABINOSE	792.7	45.0	12.6
2,4-Di-O-METHYLGALACTOSE	47.3	21.0	3.0
3,O-METHYLARABINOSE	257.1		8.8
2,-O-METHYLGALACTOSE	166.2	-	-
ARABINOSE	445.4	5.0	-
GALACTOSE	732.3	5.0	-

TABLE XXVII

probably explained by the fact that it could be separated on paper without any overlap with other materials.

Comparison of the results from the initial polysaccharide (Fisher), with those from the partially desulphated polysaccharide, confirms that much of the galactose and xylose was lost during the desulphation and methylation. The reason for this might be that these two sugars, after the desulphation, were present as "non-dialysable fragments" which were selectively lost during methylation leaving the polymeric component to pass through the procedure. It is immediately obvious, however, that the major components (ca. 85%) from the desulphated polysaccharide are the dimethylarabinose and the trimethyl-galactose, whereas, from the initial polysaccharide, monomethyl-galactose, arabinose, and free sugars constitute the major fractions. Much of the 2-O-methyl arabinose in the initial material becomes the 2,3-di-O-methyl arabinose in the desulphated polysaccharide, further proof that the majority of the sulphate linked to arabinose is situated on C-3. Furthermore if all the 2-O-methylarabinose calculated (from g.l.c.) to be present in fraction Q is sulphated at C-3 a total sulphate content of about 4.3% is required (of fraction Q; SO_4^{2-} 5.6% p.118)

It follows, from the high proportion of methylated arabinose in the partially desulphated polysaccharide, that

the initial polysaccharide must contain chains of at least 8 1,4-linked arabinose units and these may be linked together by single 1,6-linked or 1,3,6-linked galactose units. The partial acid hydrolysis studies rule out the possibility that the arabinose is present as a backbone with the galactose and xylose entirely present as side chains. That the majority of the xylose left in the partially desulphated polysaccharide is present as end group xylose, proves conclusively that it is part of the heteropolymeric material and not present as a separate xylan.

If the ratio of inter-residue galactose to arabinose (1:8) found in the two pentasaccharides isolated from the autohydrolysate, is compared with the proportion of these two sugars in the methylated desulphated polysaccharide (in which all but a negligible quantity of the galactofuranose units were lost) they are found to be approximately equal. It follows that the majority of galactose in the latter must be present in the arabinose chains as inter-residues and, that the greater part of the xylose (all present as end-group) must be attached directly to arabinose units. This is in keeping with the proportion of disubstituted units in the methylated material.

To summarise then, partial desulphation, and methylation of the desulphated polysaccharide has confirmed that much of the xylose and galactose is very acid labile and situated on the periphery of the molecule, that the majority of the

arabinose is present in short chains of 1,4-linked units sulphated at C-3, linked together by 1,3-or 1,6-linked galactose residues and situated in the inner part of the molecule, and finally that xylose forms part of the hetero-polymeric molecule.

FINAL CONCLUSIONS

From the results of this investigation, which has confirmed many of the earlier conclusions, it is possible to visualise Cladophoran as a complex, highly branched, polydisperse, heteropolysaccharide comprising mainly arabinose and galactose with smaller amounts of xylose and trace quantities of rhamnose. All the xylose units are 1,4-linked or end-group units situated on the periphery of the molecule, some being attached to galactose and some to arabinose. Much of the galactose is also present in the outer part of the molecule, although a smaller proportion is present as interchain residues joining chains of at least eight 1,4-linked arabinose residues. The latter occur in the inner part of the molecule, and, in the native polysaccharide carry ester sulphate at C-3 and/or branch points at C-2. It also appears probable that a very small proportion of the 1,4-linked arabinose units occur in the outer portion of the molecule.

These conclusions result from earlier work, from the results of autohydrolysis, and from the methylation studies of the partially desulphated polysaccharide.

The results from the Smith's degradation are in agreement with these findings. The isolation of arabinosyl-threitol as a fragment lends additional support for the heteropolymeric nature of the molecule. The presence of threitol in this, and other fragments, has been ascribed to the cleavage of 1,5-/1,6-linked galactofuranose residues. In support of this, all the investigations point to the acid lability of a proportion of the galactose and methylation of the initial polymer[56] confirms the presence of galactofuranose units.

The large change in molecular size of the degraded polymer I, compared with the initial material, is evidence that although the majority of the cleavage occurs in the outer regions of the molecule there are a small proportion of residues in the inner part that are vulnerable to periodate. In addition the considerable loss of galactose in the second oxidation provides evidence that the xylose and galactose units, removed in the first degradation, were extensively linked to galactose and that their removal left fresh galactose units vulnerable to periodate. This is direct evidence of the highly branched nature of the

galactose-xylose region of the molecule.

The relatively small amounts of arabinose (ca. 7% of total arabinose) cleaved during the second oxidation presumably became vulnerable through the removal of sugar units, or short side chains, during the first oxidation. In view of the isolation of the pentasaccharide, component 4, (Autohydrolysis B), comprising a chain of four arabinose units with a single 1,2-linked galactofuranose side-chain, it is tempting to suggest that it is this residue which has been removed. This cleavage of arabinose in the second oxidation may well explain the fragmentation of the molecule during the second degradation, in which half degraded polymer I was converted into fragments leaving the residual polymer, degraded polymer II, with a large molecular weight dispersity.

The third degradation appears to have little effect on the average size of the molecules, apart from reducing the molecular dispersity, indicating that the smaller molecules were reduced to fragments and additional degradation occurs in the larger molecules.

It should be emphasised that the Smith's degradation procedure coupled both with quantative g.l.c. analysis of the products, and with gel column chromatography to deter-

mine the relative size of the degraded polymers, provides a simple system for comparison of a series of complex polysaccharides from groups of algae. It, thus, represents an excellent system for gathering chemotaxonomic information.

A P P E N D I X

APPENDIX EXAMINATION OF SOME ENZYMES PRESENT IN CLADOPHORA RUPESTRIS

Living weed (5 Kg wet weight) was collected from Penmon Point, Anglesey, at low tide. The weed was minced in a Kenwood Chef mincer and placed in buffer (2.5% sodium carbonate, pH = 8.4, 0°C) within 8 hours of collection. The total volume of buffer (5L) was divided into two portions one of which was treated with polyvinylpyrollidone[32] (PVP-360) to a concentration of 2%.

After leaving the minced weed for an hour to extract all soluble protein the solutions (PVP and non-PVP) were centrifuged to remove cell debris and the supernatant recovered. The solution was brought to a concentration of 50% saturation with respect to ammonium sulphate and left twelve hours. The solutions were centrifuged and the sediments collected (50% and PVP 50%). The supernatant was treated with ammonium sulphate to a concentration of 100% with respect to ammonium sulphate. After 12 hours the solutions were centrifuged and the sediments recovered.

Each of the sediments from both precipitation processes was dissolved in water containing 0.005% sodium chloride and dialysed against the same solution. Finally the solutions were freeze-dried and stored in air tight bottles inside a vacuum desiccator at 0°C.

To determine whether any activity existed in the preparations each was tested against a series of carbohydrate substrates, incubating for 48 hours at 25°C in the same dilute sodium chloride buffer. The incubates were analysed by thin layer chromatography using cellulose plates with double development in solvent J. Examination of the PVP-50% and 100% extracts for sulphatase activity was also carried out and the results are shown below (TABLE XXIII) with those of the carbohydrase activity tests.

SUBSTRATE	50%	50% PVP	100% PVP
Meliobiose	-	-	-
Cellobiose	-	-	-
Laminaribiose	-	⊕	-
Maltose	⊕	⊕	-
Sucrose	+	+	-
Amylose	-	-	-
Amylopectin	-	-	-
Dextran	-	-	-
SO_4^{2-} Hydrolysis*	-	-	-

\+ = HYDROLYSIS

⊕ = HYDROLYSIS AND GLYCOSYL TRANSFER

* = ESTIMATED USING THE TURBIDOMETRIC METHOD OF REES[111] AND BY USE OF THE pH-STAT.[112]

TABLE XXIII

The results are similar to those obtained by earlier workers[113,114] with the exception that this is the first time invertase activity has been observed. This type of activity is very much to be expected since Percival and Young[115] separated sucrose and a sucrose terminated fraction from the low molecular weight fraction from Cladophora rupestris.

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The Water-soluble Polysaccharides of *Cladophora rupestris*. Part III.[1] Smith Degradation

By **P. G. Johnson** and **Elizabeth Percival,*** Department of Chemistry, Royal Holloway College, Englefield Green, Surrey

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The Water-soluble Polysaccharides of *Cladophora rupestris*. Part III.[1] Smith Degradation

By P. G. Johnson and Elizabeth Percival,* Department of Chemistry, Royal Holloway College, Englefield Green, Surrey

Application of three successive Smith degradations to the water-soluble polysaccharide of *Cladophora rupestris* has confirmed both the highly branched nature of the molecule and the presence of 1,4-linked and end-group xylose. It has provided evidence of 1,6-linked and/or 6-sulphated galactofuranose units. Proof of mutually linked arabinose and galactose units has been obtained for the first time.

EARLIER studies[1,2] on the starch-free, water-soluble sulphated polysaccharide from *Cladophora rupestris* have established that it contains approximately 20% half-ester sulphate, 8—20% protein, and galactose, arabinose, and xylose in the approximate molar proportions of 3 : 3·5 : 1 respectively. During our studies it became apparent that there is probably a family of polysaccharides which are built on the same general pattern, but which differ slightly in the proportion of the individual sugars, ester sulphate, and protein. Methylation studies[2] gave rise to a complex mixture of methylated sugars from which 2,4-di-*O*-methyl-, and 3-*O*- and 2-*O*-methyl-arabinose were separated and characterised; the last named compound was the methylated arabinose present in the largest amount. Free galactose, 2-*O*-methyl-, 2,4-di-*O*-methyl-, and 2,3,5- and 2,3,4-tri-*O*-methylgalactoses were also identified. The xylose appeared to be present solely as end-group and 1,4-linked units.

Partial hydrolysis[1] led to the separation of galactose 6-sulphate, arabinose 3-sulphate, 1,4/5-arabinobiose 3-sulphate, and 1,6- and 1,3-linked galactobioses.

All attempts to fractionate the material into separate homo-polymers failed and it was concluded that it is a hetero-polymer. Nevertheless, efforts to separate oligosaccharides containing more than a single type of sugar failed. The present paper is concerned with repeated Smith degradation (sequential periodate oxidation, reduction, and mild hydrolysis) of the polysaccharide. A single Smith degradation removes all the residues in the molecule which contains α-glycol groups and new vicinal hydroxy-groups are exposed in the degraded polymer. Repeated degradation therefore probably removes successive layers of residues from the periphery of the molecule.

It was hoped that this would yield further information on the structure of the molecule and at the same time give fragments comprising derivatives from more than a single type of sugar.

The starch-free polysaccharide, isolated as in previous experiments,[1] was subjected to three successive degradations and the yields of the various products are given on the flow diagram. The relative molar proportions of unoxidised sugars, glycerol, and threitol in each of the polyalcohols and degraded polysaccharides are also given in the flow diagram. These were determined by hydrolysis, followed by conversion into the respective trimethylsilylated (TMS) derivatives, and measurement of the peak areas obtained for these derivatives on g.l.c. These were then related to calibration curves derived from absolute weights of pure

Flow diagram of repeated Smith degradation

Polysaccharide (6·5 g.), SO_4^{2-}, 22%
Gal : Ara : Xyl
1 : 1 : 0·44

(1) IO_4^- Oxidn. (2) BH_4^- | 0·36 mole IO_4^-/Anhydrohexose unit

Polyalcohol I (6·53 g.)
Gal : Ara : Gly : Thre
1 : 1·2 : 0·40 : 0·12

H^+

Syrupy fragments (1·27 g.)

Degraded Polysaccharide I (4·53 g.)
Gal : Ara
1 : 1·9

(1) IO_4^- (2) BH_4^- | 0·24 mole IO_4^-/Anhydrohexose unit

Polyalcohol II (4·20 g.)
Gal : Ara : Gly : Thre
1 : 3·5 : 1·3 : 0·15

H^+

Syrupy fragments (1·7 g.)

Degraded Polysaccharide II (1·70 g.)
Gal : Ara
1 : 2·8

(1) IO_4^- (2) BH_4^- | 0·18 mole IO_4^-/Anhydrohexose unit

Polyalcohol III (1·63 g.)
Gal : Ara : Gly : Thre
1 : 2·36 : 0·94 : 0·28

H^+

Syrupy fragments (0·85 g.)

Degraded Polysaccharide III (1·13 g.), SO_4^{2-} 39%
Gal : Ara
1 : 2·74

The figures under Gal, Ara, Xyl, Gly, and Thre are the respective molar proportions of galactose, arabinose, xylose, glycerol and threitol in the different products. Apart from glycolaldehyde and trace quantities of xylose and rhamnose these are the only carbohydrates and derivatives detected in the different products.

[1] Sir Edmund Hirst, W. Mackie, and Elizabeth Percival, *J. Chem. Soc.*, 1965, 2958 is considered to be Part II.

[2] I. S. Fisher and Elizabeth Percival, *J. Chem. Soc.*, 1957, 2666.

standards run under the same conditions. The retention times relative to threitol are glycerol 0·41, arabinose 1·64 and 1·95, xylose 2·77 and 3·77, and galactose 4·02 and 5·33. Except for a slight overlap of the slower xylose peak with the faster galactose peak the complete separation of the other peaks facilitated the measurement and determination of the molar porportions of the constituents in the different hydrolysates.

Preliminary experiments revealed that mild hydrolysis of the polyalcohols with dilute sulphuric acid followed by neutralisation with barium carbonate resulted in considerable loss of the fragments and also of the degraded polysaccharides, possibly due to absorption on the precipitated barium salts. For this reason hydrolysis was achieved by partial conversion of the polyalcohols into their free-acid form with IR 120 H^+ resin. Chromatographic analysis of the fragments and of a hydrolysate of the recovered, degraded polymer showed the absence of free sugars in the former, and of glycerol and threitol in the latter. This was taken as evidence that the hydrolysis of the polyalcohol had not cleaved any of the glycosidic linkages and that any acetal linkages remaining unbroken are in the fragments, not present in the residual polysaccharide.

The overall results are similar in some respects to those of the Barry degradation of this polysaccharide.[3] Practically all the xylose was oxidised in the first oxidation and the trace of rhamnose in the original material increased relatively as the other sugars were oxidised. In the present experiments, however, the reaction was terminated when the primary oxidation was complete, and the proportion of galactose cleaved in the first oxidation was considerably less than in the Barry degradation where considerable over-oxidation occurred. In addition, in the latter process, due to contamination with excess phenylhydrazine, it proved impossible to separate and identify any of the fragments, apart from glyoxal. In the present experiment which is a much cleaner technique the fragments were separated (see flow-sheet for yields) and analysed by paper chromatography and g.l.c. The presence of a considerable proportion of free glycerol in each series of fragments prevented their isolation as solids and even freeze-drying produced a thick syrup. For this reason the weights recorded for the fragments must be accepted with some reserve.

From the first degradation, in addition to glycerol and threitol, five non-reducing fragments were detected. In a separate experiment the threitol was separated by paper chromatography from a total hydrolysate of the polyalcohol. It was found to be contaminated with a small amount of rhamnose which has the same chromatographic mobility. The latter was oxidised to rhamnonic acid and separated from the threitol on an anionic resin, whereupon the threitol crystallised. It was converted into the crystalline 1,3-benzylidene derivative.[4]

Glycerol in the fragments could be derived from adjacent 1,4-linked xylose and arabinose and also from 1,6-linked or 6-sulphated end-group galactose. D-Threitol could only arise from galactose, 1,6-linked 4-sulphated pyranose, 1,4-linked pyranose, or 1,6-linked or 6-sulphated furanose residues. In view of the earlier methylation and partial hydrolysis results, the furanose derivatives appear to be the more probable precursors of the threitol. Calculation of the relative molar weights revealed that the amount of threitol produced in the first and third degradation corresponded to the greater part of the galactose oxidised, but that in the second degradation the threitol represented only about a third of the galactose oxidised. From this it may be concluded that not all the galactofuranose units are present in the periphery of the molecule.

In addition to glycerol and threitol three other neutral and two acidic fragments were separated by ionophoresis followed by paper chromatography. The first of these on hydrolysis gave arabinose and threitol and the second galactose and glycerol, both in the molar ratios of 1 : 1. The rest of these fragments contained galactose, arabinose, glycerol, and threitol, and since these were not in strict molar proportions it seemed very probable that they were mixtures; it was, therefore, impossible to draw any valid conclusions concerning their structure.

The most important fragment is the arabinosylthreitol since it provided the first direct proof of the heterogeneity of the polysaccharide in that both galactose and arabinose are present in the same molecule. Although the amount of this fraction separated as a pure substance was only sufficient to permit the characterisation of the constituents by paper chromatography and g.l.c., their characterisation as crystalline materials from a total hydrolysate is considered conclusive proof of their identity.

In the subsequent degradations, apart from some glycerol and threitol, all the fragments carried ester-sulphate groups. Chromatography indicated a complex mixture of substances, and only two fractions separated from the third degradation could be regarded as pure substances. Both contained galactose, arabinose, glycerol, and threitol. Since each of these fractions gave a single discrete spot on a paper chromatogram, with the mobility of a tetra- and a higher oligosaccharide, and a single discrete spot on ionophoresis it is difficult to imagine that they can be mixtures, particularly since their constituents are present in molar proportions. Earlier Smith degradation studies on a sulphated glucuronoxylorhamnan [5] have provided evidence of the stabilising effect of the ester-sulphate groups on neighbouring acetal-linkages, and it seems probable that under the mild conditions of hydrolysis some of the acetal linkages escape hydrolysis and single fragments containing both arabinose and galactose and threitol and glycerol linked together have been hydrolysed from

[3] J. J. O'Donnell and Elizabeth Percival, *J. Chem. Soc.*, 1959, 1739.

[4] A. B. Foster, H. Haines, and J. Lehmann, *J. Chem. Soc.*, 1961, 5011.

[5] Q. N. Hag and Elizabeth Percival, in 'Proceedings of the Vth International Seaweed Symposium,' ed. E. G. Young and J. L. McLachlan, Pergamon Press, Oxford, 1966, p. 261.

the parent polyalcohol. If this is so then it provides additional evidence of the heterogeneity of the polysaccharide.

The comparatively high reduction of periodate in the second and third oxidations and the variety of fragments set free at each degradation is strong evidence in favour of a highly branched molecule.

Although the present results permit no extensive deductions about the internal structure of the molecule it is expected that the information obtained will be of value in future studies.

EXPERIMENTAL

Paper chromatograms were eluted with butan-1-ol–pyridine–water (6 : 4 : 3), sprayed with, (1) a saturated solution of aniline oxalate, and then heated at 120° for *ca.* 5 min., or (2) a 1% solution of silver nitrate in acetone, followed by 0·5N-sodium hydroxide in 70% ethanol, then 2% sodium thiosulphate (the papers were dipped in each solution and dried at room temperature between each operation), or (3) a 0·1% solution of Bromocresol Green in ethanol, and then alkali was added until the solution was just blue. Electrophoresis was carried out in pyridine–acetic acid buffer, pH, 7·0 at 2000v and 30 mA for 1·5 hr. R_{gal} is the chromatographic mobility of the sugars relative to galactose. M_{galA} is the distance migrated by the sulphated fragments if the D-glucose spot is taken as the starting point divided by the distance between D-galacturonic acid and D-glucose spots. G.l.c. was carried out on a Pye Argon Chromatograph in which argon was employed as the mobile gas-phase. The stationary liquid-phase was 10% by weight of Apiezon K supported on Celite at 155° for the glycerol and threitol and 175° for the threitol and the sugars. The carbohydrate content was determined by the phenol–sulphuric acid method [6] and the quantities were read off graphs constructed from solutions containing the appropriate sugars in the appropriate proportions. Acid hydrolyses were carried out with 90% formic acid in sealed tubes in an atmosphere of carbon dioxide for 6 hr. at 100°; the mixtures were then diluted with water (4 vol.) and heated for an hour. Sulphate (expressed as percentage of carbohydrate) was determined by the method of Jones and Letham [7] after digestion of the polysaccharide (10 mg.) with concentrated nitric acid (AnalaR) (1 ml.) and a few mg. of sodium chloride in a sealed tube for 18 hr. at 100°. The acid was then evaporated off and the dry tube was heated in an oven at 110° for 2 hr. The contents were then dissolved in water (1 ml.) and appropriate aliquots were removed for the determination. The degraded polysaccharide III was extensively dialysed before analysis for sulphate.

The starch-free, sulphated, water-soluble polysaccharide was isolated as before.[1] It had $[\alpha]_D$ +56° and contained 50% carbohydrate, 22% sulphate, 13·5% ash, and 12·5% protein. The relative molar proportions of galactose, arabinose, and xylose are given in the flow diagram.

Periodate Oxidation.—The polysaccharide (6·5 g.) was oxidised with 0·015M-sodium periodate (2 l.) at room temperature in the dark and aliquots (0·5 ml.) were withdrawn at intervals, and the reduction of periodate was measured.[8] The primary oxidation was complete after 5 hr. when 0·36 moles of periodate had been reduced for every C_6 anhydrounit. The reaction was stopped by destruction of the excess periodate with ethylene glycol; the polyaldehyde (6·4 g.) was recovered as a white solid by precipitation with ethanol (1 vol.).

Reduction with Borohydride.—To the polyaldehyde (6·3 g.) in 0·05M-boric acid (500 ml.) potassium borohydride (10 g.) was added and the reduction was allowed to proceed at room temperature for 48 hr. during which time the pH never rose above a value of 9—10. The polyalcohol (6·53 g., containing 2·5 g. of unoxidised sugars) was recovered as a white solid by precipitation with ethanol (1 vol.). Analysis by paper chromatography of a total hydrolysate of a portion revealed galactose, arabinose, glycerol, and threitol together with traces of xylose and rhamnose.[3]

Characterisation of the Threitol.—In a separate experiment the polyalcohol (500 mg.) was hydrolysed and the derived syrup was separated on Whatman No. 17 paper.[9] The portion of paper containing threitol was eluted with water. After concentration of the eluate trace quantities of rhamnose were also found. The mixture, dissolved in water, was oxidised with bromine at room temperature for 48 hr. The threitol was then separated from the rhamnonic acid on a column of Amberlite IRA400 (OH^-) resin. The threitol was eluted with water and on concentration of the eluate it crystallised, m.p. 88°. It was converted into the crystalline 1,3-monobenzylidene derivative, m.p. 130°,[4] (from toluene). Its TMS derivative gave a single peak on g.l.c. with the same retention time as the TMS derivative of authentic 1,3-monobenzylidene L-threitol run as standard.

Autohydrolysis of the Polyalcohol.—To the polyalcohol (6·5 g.) dissolved in water (500 ml.) Amberlite IR 120 (H^+) resin was added with stirring until the pH dropped to a value of 1·5; the stirring was continued at room temperature for 24 hr. The resin was then filtered off and the solution was neutralised with ammonia. The degraded polymer was precipitated with ethanol (1 vol.) and separated by centrifugation. It was dissolved in water and freeze-dried to a white solid (4·53 g. containing 2·0 g. of sugar). The alcoholic supernatant solution was concentrated to small volume and the derived aqueous solution was freeze-dried to a thick syrup (1·27 g., containing 0·24 g. of unoxidised sugar). Paper chromatography (spray 2 and 3) revealed the presence of glycerol and threitol together with spots with the mobilities R_{gal} 1·22, 1·0, 0·97, 0·65, 0·47, and 0·2—0·4. Spray 3 and ionophoresis revealed two sulphated fragments with M_{galA} 1·33 and 1·00.

Separation of Fragments.—The syrup was separated into neutral and acidic fragments by ionophoresis (12 papers) and elution of the appropriate areas, followed by further separation on Whatman No. 17 and No. 1 papers. The free threitol and glycerol were discarded.

Fraction 1 was a neutral syrup, R_{gal} 1·22, which on hydrolysis gave arabinose and threitol (paper chromatography, spray 2). Quantitative g.l.c. of the TMS derivatives of the hydrolysate gave the respective molar proportions of 1 : 1.

Fraction 2 was a neutral syrup, R_{gal} 0·97. A hydrolysate revealed galactose and glycerol (paper chromatography, and g.l.c. of the TMS derivatives gave the molar proportions of 1·0 : 1·0.

Fraction 3 was a neutral syrup, R_{gal} 0·47, which examined

[6] M. Dubois, K. A. Gilles, J. K. Hamilton, P. A. Rebers, and F. Smith, *Analyt. Chem.*, 1956, **28**, 350.
[7] A. S. Jones and D. S. Letham, *Chem. and Ind.*, 1954, 662.
[8] G. O. Aspinall and R. Ferrier, *Chem. and Ind.*, 1957, 1216.
[9] Harriet L. Frush, *J. Res. Nat. Bur. Stand.*, 1967, **71**, *A*. 49.

as for the above fraction gave galactose, arabinose, glycerol, and threitol in the molar ratio of 1 : 1·3 : 1·2 : 1.

Fraction 4 was an acidic syrup R_{gal} 0·47 (spray 2 and 3), M_{galA}, 1·0, which gave on hydrolysis galactose, arabinose, glycerol, and threitol in the molar ratio of 1 : 0·72 : 1·5 : 0·8.

Fraction 5 was an acidic syrup, R_{gal} 0·2—0·4 (spray 2 and 3), which on hydrolysis gave galactose, arabinose, glycerol, and threitol in the molar ratio of 1 : 1·5 : 1·7 : 0·3.

Subsequent Degradations.—The degraded polysaccharide I (4·5 g.) was subjected to two further successive Smith degradations under exactly the same conditions. The primary oxidation was complete in 5·5 hr. in the second and in the third in 14 hr. The respective recovery of unoxidised sugars were polyalcohol II, (1·5 g.), degraded polysaccharide II (0·65 g.), polyalcohol III (0·56 g.), and degraded polysaccharide III (0·32 g.). Apart from glycerol and threitol all the entities in the fragments were non-reducing and sulphated. This made separation difficult and only the following two fractions gave single spots both on paper chromatography and ionophoresis.

Fraction I was an acidic syrup, R_{gal}, 0·5, M_{galA}, 1·0, and contained galactose, arabinose, threitol, and glycerol in the molar ratio of 1·0 : 1·9 : 0·93 and 2·0 when analysed in the same way as the earlier fractions.

Fraction II was an acidic syrup, R_{gal} 0·4, M_{galA} 1·82 and contained galactose, arabinose, threitol, and glycerol in the molar ratio of 1 : 1 : 0·9 : 1·0.

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