Structural investigation of the extracellular polysaccharides metabolised by S19, a Xanthomonas type bacterium and by the unicellular red alga, *Rhodella maculata*

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This thesis is dedicated to the late

Professor Edward John Bourne
ABSTRACTS

Part I

Structural studies on the extracellular polysaccharide elaborated by the bacterium, Xanthomonas S19, have been made. The component monosaccharides, D-glucuronic acid, D-glucose, D-galactose, and D-mannose were characterised and their approximate molar ratios of 1.6 : 3 : 1 : 1 determined. Attempted fractionation by a number of methods was unsuccessful and it is concluded that it is a single polydisperse acidic heteropolysaccharide. Partial hydrolysis studies confirmed this conclusion and indicated a repeating structure of 13 residues including all the above monosaccharides.

Acetate esters were detected and confirmed and account for a degree of acetylation equal to 2. They were shown to be located on C-2 and/or C-3 of the glucose residues.

The linkages of the sugar units were determined by methylation studies: the glucose is linked as non-reducing end-group and also as 1,2- and 1,4-linked units; the galactose units are solely 1,3-linked; the glucuronic acid residues are 1,4-linked; a major proportion of the mannose units are 1,2,4-linked and the rest 1,2-linked.

The similarities and differences between this polysaccharide and those from other Xanthomonas species are discussed.
Part II

The extracellular mucilage metabolised by the microscopic unicellular red alga, *Rhodella maculata*, has been investigated. The constituent sugars xylose, glucuronic acid (both major), 3-O-methyl xylose, rhamnose, galactose and glucose have each been characterised. This is the first time 3-O-methylxylose has been found as a constituent of a natural polysaccharide. Protein to the extent of 16-26\% is also present and 15 amino acids have been identified. All attempts to fractionate the material into more than a single polysaccharide were unsuccessful and gel electrophoresis showed only one band for polysaccharide and this was free from protein. Half ester sulphate groups are present to about 16.5\% of the carbohydrate and tentative evidence is advanced that these may be linked to the glucuronic acid.

Methylation and periodate oxidation studies proved that the xylose units are 1,4- and 1,3-linked, some of the 1,4-linked units carrying methoxyl at C-3. The glucuronic acid occurs as 1,3-linked units. Rhamnose is present as end-group together with 1,3- and 1,4-linked galactose, and 1,2- and 1,4-linked glucose. Branch points are occupied by 1,3,4- and 1,2,4-linked galactose and/or glucose units.

The difference of this heteropolysaccharide from the characteristic galactans of the red algae is discussed.
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PUBLICATION
Part One

Structural investigation of the extracellular polysaccharide metabolised by S19, a Xanthomonas type bacterium.
GENERAL INTRODUCTION

1. Introduction to Bacteria
   
   A: Ecology and role of bacteria in Nature

   Bacteria are found almost everywhere. Survival within the vast number of habitats results in an enormous variety of nutritional and physiological types. They are abundant in soil, water and air. Since they are usually carried by water or on dust particles, their distribution is world-wide, although there are some unique parasites dependant on special carriers such as insects.

   A number of bacteria possess enzymes that are capable of breaking down such large molecules as cellulose, starch, lignin, fats, and protein into simpler substances. Some of the carbon dioxide utilised by plants is released into the atmosphere by the action of bacteria on dead organic matter. On the other hand, certain bacteria make it possible for plants to utilise atmospheric nitrogen (nitrogen fixation); the bacteria best known for this process include species of Rhizobium, Azotobacter, and Clostridium.

   The relation of bacteria to other living things is also seen through the mutual association of cellulolytic bacteria with grazing animals and of luminous bacteria with certain deep-sea fishes. The harmful effect of pathogenic bacteria to plants and animals is of great economic importance to man.

   B: Characterisation of bacteria:— morphology, anatomy and physiology

   Bacteria are unicellular and are among the smallest living organisms known. They are usually about 0.3 - 2 μ in diameter,
although they can be as long as 10 - 15 μ. Most bacteria possess an outer cell wall which confers mechanical rigidity upon the cell, and is responsible for the cell's characteristic shape. This may vary from spherical to rod- or spiral-shaped cells. The morphology of the bacterial cell may be divided as follows:

1. **Surface appendages**: These are the flagella, which are responsible for locomotion, and the fili or fimbriae which are responsible for adhesion. These are composed of flagellin, a protein, related chemically and physically to the hair-like proteins of other organisms.

2. **Surface adherents**, for example, capsules and slime layers:– Outside the rigid cell wall, some bacteria are surrounded by gelatinous material. The distinction between capsule and slime layer is very arbitrary and depends largely upon their relative viscosity, and other solution properties. The division is made more complicated by the presence of very small amounts of surface materials which are firmly bound to the cell wall. These various layers may be removed by specific enzymes.

The capsules and slimes are composed of polysaccharide and polypeptide. The carbohydrates are composed of hexoses, pentoses, uronic acids, or amino sugars joined by a variety of linkages. The capsules of *Bacillus anthracis* and *B. megaterium* contain in their polypeptide chains D-glutamic acid linked through its gamma-carboxyl group. Such a structure has not been found in cells of higher plants and animals, and seems to be unique to bacteria.
3. **Surface layers:** These include the rigid cell wall, and the immediately underlying protoplasmic membrane, the plasmalemma.

The cell wall is the major skeletal structure of the bacterial cell and enables a division of bacteria into Gram-positive and Gram-negative species. This is dependant upon marked differences in the cell wall composition. Gram's stain contains crystal violet and iodine which form a violet-coloured complex with cell wall material. The complex is not extracted by alcohol and acetone from Gram-positive cells, and these appear violet. The complex is extracted from Gram-negative cells, which stain red when the counter-stain Safranin is added.

The cell walls contain the most distinctive bacterial products. The major structural component is a peptidoglycan, which is responsible for mechanical rigidity. This forms the basic building blocks of both Gram-positive and Gram-negative cell walls. The glycan portion is made up of alternating $\beta-1,4$-linked units of $N$-acetylmuramic acid and $N$-acetylglucosamine arranged in linear chains. Virtually all of the carboxyl groups of the $N$-acetylmuramic acid residues are involved in amide linkages to terminal L-alanine residues of the peptide moiety.

![Chemical structure of peptidoglycan](attachment:image.png)

$N$-acetylglucosaminy1-$\beta$-1,4-$N$-acetylmuramic acid
The normal range of variety and isomeric forms of amino acids is present in Gram-negative bacteria whereas only a few major amino acids are present in Gram-positive forms. This difference in amino acid composition is responsible for their different behaviour towards Gram's stain.

Some amino sugars found in bacterial cell walls are of very limited distribution in Nature. For example, muramic acid is confined to the bacteria, blue-green algae and rickettsiae. The amino acid, diaminopimelic acid, \((\text{HOOC} \cdot \text{CH(CH}_2)_3 \cdot \text{CH} \cdot \text{COOH})\), first detected and isolated from the bacterium *Corynebacterium diphtheriae* in 1950, was subsequently found to be one of the characteristic cell wall amino acids of many bacterial species. Another unusual feature is the presence of a number of amino acids as D-isomers, e.g., alanine, glutamic acid, aspartic acid and phenylalanine.

In the Gram-positive bacteria, teichoic acids and polysaccharides may be covalently linked to the peptidoglycan. The occurrence of teichoic acids has so far only been reported in bacteria. They are polymers of ribitol or glycerol phosphate with additional compounds such as glucose, N-acetylglucosamine and D-alanine linked to the polyol backbone, e.g. the teichoic acid from the cell wall of *Staph. lactic* consists of

\[
\begin{align*}
\text{CH}_2 \text{OH} & \quad \text{R} = \text{H} \\
\text{HO} & \quad \text{CHO} \\
\text{NH}_2 & \quad \text{C}_n \text{H}_2 \text{O}\text{P}_n \text{CH}_2 \text{OH} \\
\end{align*}
\]
The polysaccharides contain mainly glucose, galactose, mannose, arabinose, rhamnose, a heptose and hexosamine. In the Gram-negative bacteria, lipopolysaccharide-protein-lipid complexes may be attached to the peptidoglycan, and present a more complex structure. The bacterial lipopolysaccharides contain a unique class of sugars, the dideoxy sugars, 3:6-dideoxy-L-galactose (colitose), 3:6-dideoxy-D-galactose (abequose), 3:6-dideoxy-D-glucose (paratose), and 3:6-dideoxy-D-mannose (tyvelose).

The plasmalema is the site of the cell's osmotic barrier, and a number of enzymes are localised almost exclusively in this membrane. Virtually all of the cell's lipid, phospholipid, carotenoid, and cytochromes (electron-transport components) are located in the plasmalema, and its intrusion into the cell, called the mesosome.

The plasmalema of some Gram-positive bacteria can be obtained from their cells by first dissolving their cell walls with lysozyme to give the intact protoplasts, followed by separation after osmotic lysis of the protoplasts, these being the portions of the cells within the cell walls. It is made of about 60% protein, 20% lipid, and 20% carbohydrate. The chemical composition of bacterial plasmalemas is thus similar to membranes of higher plant and animal cells. So far it has not been possible to obtain the protoplast of Gram-negative bacteria.

4. Cytoplasm, intracellular organelles, particles and granules: The cytoplasm contains soluble enzymes, and cell solutes such as inorganic ions, and various amino acids, carbohydrate precursors, purines, pyrimidines and other substances constituting a metabolic pool.
The principal intracellular structure, the bacterial nucleus, is procaryotic (not membrane bound), and contains deoxyribonucleic acid (DNA). In addition it also contains ribonucleic acid (RNA) and protein. The nucleus carries the pattern material for forming new cells and hereditary genes.

Among the particles and granules are the ribosomes (RNA-protein particles) which are the active sites of protein synthesis; the chromatophores (the pigment-containing particles of photosynthetic bacteria); lipid granules and various polysaccharide granules.

5. Special structures such as the endospore and stalk:-
Spores are formed in some genera when the environmental conditions become unfavourable for active metabolism and cell reproduction. They are more resistant to heat, drying, light and disinfectants than the original cell. They possess a multilayered wall with a complex amino acid composition. They contain a large amount of dipicolinic acid, pyridine-2-6-dicarboxylic acid, a substance so far only encountered in Nature in bacterial spores.

C: Bacterial Taxonomy

The taxonomy of bacteria is in a far less developed state than that of the higher animals and plants. According to Bergey's manual of determinative bacteriology the bacteria are classified as Schizomycetes, a class of fungi, although it is now clear that bacteria are procaryotic organisms. The classification of bacteria into subgroups and smaller divisions presents some difficulties since the number of morphological characteristics is limited and these may vary in different environments. However, their diverse physiological characteristics in conjunction with their morphological
characteristics are used in separating bacteria into their orders, families, genera, and finally the species.

Bacteria are related to the protozoa, a large group of unicellular animals that include the amoeba, and paramecium. In many respects the bacteria resemble some of the simpler plants, particularly the blue-green algae, and some of the moulds. Other related groups are the rickettsiae and viruses.

D: Multiplication of bacteria

Reproduction in bacteria is primarily asexual and occurs by fission of the cells. It is very rapid and a cell may divide every 20 or 30 minutes. Hence, the growth of bacterial cultures in a favourable environment is exponential with time (lag phase). This growth is preceded by a log phase and terminated by the stationary phase. Other modes of reproduction include, conidia cell formation, and sexual reproduction.

Gene transfer from one bacterial cell to another has been found to occur in a limited number of species. This is brought about by transformation, transduction, or conjugation. Transformation is the transfer of soluble DNA from one type of cell to another. Transduction is the transfer of genetic material by a particle of bacteriophage and conjugation involves direct cell contact with temporary cell fusion.

Variations in bacteria are in most cases inherited through gene mutation. However direct mutation may occur from interaction of physical or chemical agents with the DNA molecule. The new hereditary pattern is then transmitted through many generations.
E: Metabolism of bacterial polysaccharides

Anabolism.

The producers of carbohydrates in Nature are the plants. They do so by the process of photosynthesis. The overall process is more complicated than is indicated by the following simple equation:

\[
\text{CO}_2 + \text{H}_2\text{O} \xrightarrow{\text{light}} (\text{CH}_2\text{O}) + \text{O}_2
\]

\[\Delta G^0 = +115 \text{ kcal mole}^{-1}\]

With the exception of bacteria, all other life-forms that are unable to photosynthesise carbohydrates depend directly or indirectly on plants. The few types of bacteria that can produce their own carbohydrates do so either by photosynthesis or chemosynthesis. Photosynthetic bacteria can be either autotrophic or heterotrophic. Chemosynthetic bacteria can be either aerobic, anaerobic, or fermentative.

It is now well established that the synthesis of bacterial polysaccharides are biochemical reactions involving enzymes. Whether extracellular polysaccharides are produced by extracellular enzymes, or whether the polymer is formed intracellularly and then excreted is still not clear. Bacterial metabolism has been extensively covered by Doelle. More specifically, the synthesis of polysaccharides has been covered by Z.W. Hassid.

The biosynthesis of complex saccharides (oligosaccharides, glycosides, and polysaccharides) involves the process of trans-glycosylation. In this process, the glycosyl donor may be sugar phosphate, sugar nucleotide, oligosaccharide or polysaccharide.
Transglycosylation can be represented by the equation:

\[ \text{G1–O–X} + \text{E} \rightarrow \text{G1–O–E} + \text{X}, \]

where G1–O–X is a glycosyl donor in which G1 is the sugar unit, X is the aglycone and E is the transglycosidase (see below for illustration).

It has been found that the nucleoside diphosphate sugars are the most effective donors of the glycosyl moiety for complex formation. This is in accordance with the thermodynamics of the reaction: since nucleoside diphosphate sugars have the highest negative free energy of hydrolysis of all known compounds containing glycosyl groups that can serve as a monosaccharide donor. A few examples will serve to illustrate the various enzymic intermediates and their interrelation in the biosynthesis of complex saccharides.

1. Formation of a nucleoside diphosphate sugar.

Nucleoside triphosphate + sugar-1-phosphate

\[ \text{Nucleoside triphosphate} + \text{sugar-1-phosphate} \]

\[ \xrightarrow{\text{phosphorylase}} \]

nucleoside diphosphate sugar + pyrophosphate e.g.:\(^2\)

GTP (Guanine triphosphate) + α-D-glucopyranosyl phosphate

\[ \xrightarrow{} \]

GDP-D-glucose + PPi

2. GDP-D-glucose + acceptor

\[ \text{GDP-D-glucose} + \text{acceptor} \]

\[ \xrightarrow{\text{glycosyltransferase}} \]

Accepter-glucose + GDP

\[ \xrightarrow{\text{repeated n times}} \]

β(1→4)-D-glucan (cellulose).
3. UDP-D-glucose + acceptor \[\text{glycosyltransferase}(n \text{ times})\] \[\beta(1\rightarrow 3)-D-glucan.\]

4. UDP-D-glucose \[\text{dehydrogenase}\] UDP-D-glucuronic acid

\[\text{acceptor} \rightarrow \text{glycosyltransferase}\]

\[\beta-D-Glc-(1\rightarrow 3)-D-GlcUA\]

i.e. alternating unit polysaccharide.

5. UDP-D-glucuronic acid

\[\downarrow \text{epimerase}\]

\[\downarrow \text{decarboxylase}\]

UDP-D-galacturonic acid

\[\downarrow \text{UDP-D-Xylose}\]

\[\downarrow \text{Xylan}\]

The biosynthesis of complex heteropolysaccharides may thus involve a system of enzymes, e.g. kinases, isomerases, phosphatases, epimerases, phosphorylases, glycosyltransferases, dehydrogenases, and decarboxylases. This multienzyme system is presumably shared, in part or wholly, by different types of bacteria. It follows that the synthetic capability of one species would therefore be very wide, unless the ability to synthesise a particular heteropolysaccharide is a stable genetic character, and hence indicating that DNA provides the initial replica for synthesis, under appropriate growth conditions.
Sulphate residues have been shown to be transferred directly from the active sulphate donor (e.g., PAPS, adenyl-3-phosphate) to the polysaccharide.

Methylation also occurs at the polysaccharide level. A particulate enzyme from Phaseolus aureus was shown to be capable of transferring the methyl group of S-adenosyl-L-methionine to the D-galacturonan that is present with it.

Very little is known specifically on the carbohydrate metabolism of Xanthomonas species. The closest metabolic studies to this genus are on the related genus, Pseudomonas.

2. Bacterial Polysaccharides

A: Introduction to bacterial polysaccharides

At some stage during their life-cycle, all microorganisms produce highly complex sugar macromolecules which are known as polysaccharides. The carbohydrate nature of these macromolecules has been noted since the time of Pasteur. Information has been gathered regarding the polysaccharides of Gram-positive bacteria, Gram-negative bacteria, viruses, rickettsiac, moulds, yeasts, fungi and protoza. Bacterial polysaccharides have been investigated for a number of reasons, the most significant being their commercial value, and their importance in classification and pathogenicity. Morphologically, bacterial polysaccharides can be divided into (a) intracellular polysaccharides, (b) cell wall polysaccharides, and (c) extracellular polysaccharides. Little work has been done on the intracellular polysaccharides and these will not be discussed. The characteristics of cell wall polysaccharides have already been described and certain generalisations have been drawn upon their constitution. Similar
conclusions on the extracellular polysaccharides have been possible only for a few genera,\textsuperscript{39} perhaps, because of the difficulty in defining their exact location: they are often described as loose slime or medium soluble polysaccharides, capsular polysaccharides, or microcapsular polysaccharides. These distinctions are arbitrary since the extracellular layers are closely bound and may permeate one another. Moreover, in many cases, only the polysaccharide parts of the extracellular polymers have been investigated, when these polymers may be protein-polysaccharide complexes, or lipoprotein-containing polysaccharides.

Structurally, the extracellular polysaccharides can be divided into homopolysaccharides and heteropolysaccharides.

**B: Extracellular homopolysaccharides of bacteria**

Glucan, fructan, galacturonan, mannan, galactan, xylan and araban have all been discovered in Nature, though the bacterial extracellular homopolysaccharides are mainly glucans and fructans. The major types of bacterial extracellular homopolysaccharides are tabulated below.
### Bacterial extracellular homopolysaccharides

<table>
<thead>
<tr>
<th>Polymer</th>
<th>Monomer</th>
<th>Main linkage</th>
<th>Branching linkages (if any)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cellulose</td>
<td>glucose</td>
<td>(\beta(1\rightarrow4))</td>
<td>-</td>
</tr>
<tr>
<td>Starch</td>
<td>glucose</td>
<td>(\alpha(1\rightarrow4))</td>
<td>(\alpha(1\rightarrow6))</td>
</tr>
<tr>
<td>Dextran</td>
<td>glucose</td>
<td>(\alpha(1\rightarrow6))</td>
<td>(\alpha(1\rightarrow2))</td>
</tr>
<tr>
<td>Levan</td>
<td>fructose</td>
<td>(\beta(2\rightarrow6))</td>
<td>(\beta(2\rightarrow4))</td>
</tr>
<tr>
<td>Glucan</td>
<td>glucose</td>
<td>(\beta(1\rightarrow2))</td>
<td>-</td>
</tr>
<tr>
<td>Curdlan</td>
<td>glucose</td>
<td>(\beta(1\rightarrow3))</td>
<td>-</td>
</tr>
<tr>
<td>Mannan</td>
<td>mannosamine</td>
<td>(1\rightarrow6)</td>
<td>-</td>
</tr>
</tbody>
</table>

Cellulose is synthesised extracellularly in culture mediums by several species of *Acetobacter*. The bacterial cellulose and starch resemble plant celluloses and starches in their chemical and physical properties. Dextrans are produced by many strains of bacteria, mainly *Leuconostoc* and *Streptococcus* species. Although their main linkage is \(\alpha(1\rightarrow6)\), the proportion of the other linkages varies from one strain of microorganism to another. They may have very high molecular weight and their molecular weight dispersity is governed by previous treatments during isolation. Levans are produced by widely different organisms, including *Bacilli*, *Streptococcus salivarins* and *S.bovis*, *Pseudomonas prunicola*, and *Actinomycetes*. They are extremely labile to acid hydrolysis.

\(\beta\) 1,2-D-glucan produced by *Agrobacterium* species are discussed later. *Rhizobium japonicum* produces a similar glucan. Another glucan of unknown structure is produced by *Arthrobacter simplex*.

The water-insoluble gelatinous polysaccharide produced by a naturally occurring mutant of *Alcaligenes faecalis* is a linear \(\beta\) 1,3-D-glucan though it also contains a very few 1,6-linkages.
Different types of homopolysaccharides are produced by Neissera meningitides. The group A polysaccharide was shown to be a homopolymer of D-mannosamine 6-phosphate which is partially N- and O-acetylated. The principal glycosidic bond seems to be a (1 → 6) phosphodiester bond. The exact location of the polymer is not known although it must be close to or on the bacterial surface.

Groups B and C polysaccharides are homopolymers of neuraminic acid. Group B polysaccharide contains N-acetyl groups whereas Group C polysaccharide contains both N- and O-acetyl groups.

C: Extracellular heteropolysaccharides of bacteria

These polysaccharides are of varying complexity. The repeating unit may be a heterotrisaccharide or it may be rather more complex such as an acetylated and pyruvylated hexasaccharide composed of 4 different sugars. The component sugars of polysaccharides produced by microorganisms are listed in tabular form by Stacey and Barker. Glucose, galactose and mannose are the three commonest neutral sugars found. The rarely occurring D-form of arabinose is obtained from the polysaccharide of Tuberculin, the concentrated bacteria-free liquid culture in which tubercle bacilli have grown. A heptose has also been characterised in the extracellular bacterial polysaccharide of A. indicum as D-glycero-D-mannoheptose. Other constituents are acetyl, amino, phosphate, succinate, O-ethylidene, O-carboxyethylidene, and formate groups. As a result a great variety of polysaccharides are produced by bacteria.

Several reviews have appeared on bacterial polysaccharides. The article on bacterial polysaccharides by T.E. Taylor and H. Hibbert,
although mainly on dextrans and levans summarised the work done up to 1946. The review by J.F. Wilkinson (1958) sought to generalise our knowledge of the bacterial extracellular polysaccharides. The polysaccharides of Gram-negative bacteria were reviewed by O. Luderitz et al. (1968), and by D.A.L. Davies. The comprehensive review by I.W. Sutherland (1972) also describes bacterial exopolysaccharides. The article by B. Lindberg and S. Svennson (1973) supplements the other works. Further the literature on bacterial polysaccharides is reviewed in periodical reports of the Chemical Society. The fine structure of some of these polysaccharides will be discussed.

Colanic acid: This is the name given to the slime produced by many species in the bacterial family Enterobacteriaceae. It consists of fucose, galactose, glucuronic acid, acetate and pyruvate in the approximate molar ratio of 2:2:1:1:1. The repeating hexasaccharide unit has been reported:

\[ 4,6-O-(1'-carboxyethylidene)-D-Gal \]
\[ \downarrow 1 \beta \]
\[ GlcUA \]
\[ \downarrow 4 \]
\[ Gal \]
\[ \downarrow 3 \]
\[ 3 \text{ or } 4 \text{ Acetyl} \]
\[ \rightarrow 3)-Glc-\beta (1 \rightarrow 3 \text{ or })-Fuc-(1 \rightarrow 4)-Fuc-(1 \rightarrow ) \]

Variations in colanic acid structure are the absence of acetate or the presence of different substituents on the terminal galactose residue.
Pyruvic acid: This has been found in a number of polysaccharides. It is usually present on one component of the heteropolysaccharide, for example, glucose, or galactose, or mannose; but in the extracellular polysaccharide of Rhizobium trifolii, it is present on both galactose and glucose, as in the reported repeating structure:

\[
\begin{array}{c}
(C.Et)-D-Gal \\
\downarrow 3 \\
(C.Et)-D-Glc \\
\downarrow 4 \\
Glc \\
\downarrow 6 \\
\rightarrow 4) \rightarrow D-Glc-(\rightarrow 4) \rightarrow D-Glc-(\rightarrow 4) \rightarrow D-Glc-(1\rightarrow 4) \rightarrow D-GlcUA-(1\rightarrow 4) \rightarrow D-Glc
\end{array}
\]

\( C.Et \equiv 4,6-O-(1'\text{carboxyethylidene}). \)
Heptose: This has been found in a number of bacterial exopolysaccharides.\textsuperscript{79,80-83} D-glycero-D-mannoheptose is present in \textit{Azotobacter indicum} extracellular polysaccharide. This contains D-glucuronic acid, D-glucose and heptose in equimolar ratio and is reported to compose of the repeating trisaccharide: \textsuperscript{79} 

\[
[\rightarrow 4)-D-\text{GlcUA-(1\rightarrow 3)-D-Glc(1\rightarrow 2)-Heptose(1\rightarrow)]_n.
\]

\textit{Azotobacter vinelandii}: The extracellular polysaccharides elaborated by several strains\textsuperscript{84,85} of \textit{Azotobacter vinelandii} is a partly O-acetylated polyuronide, consisting of D-mannuronic acid and L-guluronic acid. It resembles alginic acid from brown algae\textsuperscript{86} since it contains 4-O-linked mannuronosyl and guluronosyl residues. These are distributed along the polymer chain in the typical, block-wise fashion characteristic for alginic acid.

\textit{Pneumococcus}: D-pneumonia Type III extracellular heteropolysaccharide\textsuperscript{87} consists of repeating unit of cellobiuronic acid,

\[
[\rightarrow 3)-D-\text{GlcUA-\beta(1\rightarrow 4)-D-Glc-\beta(1\rightarrow)]_n.
\]

This structure is possibly the simplest repeating unit of extracellular heteropolysaccharides of \textit{Pneumococci}, if not, of all bacteria.

3. Phytopathogenic Bacterial Polysaccharides.

\textbf{A: Polysaccharides of bacteria other than \textit{Xanthomonus} species}

The exudates produced by plant pathogens have been more of concern to botanists; and very few of these extracellular polysaccharides have been investigated by chemists. The polysaccharides produced by species of \textit{Erwinia} have hardly been examined, although, glucose was shown to be the main component of \textit{E. caratovora} exudate.\textsuperscript{88-9}
M.E. Karkenny et al.\(^{90}\) reported that the purified polysaccharide from 5 species of *Pseudomonas* gave glucose as the only sugar. Deinema et al.\(^{91}\) also noted the formation of true cellulose in amounts ranging from 1-4% by several strains of *Pseudomonas*.

*Pseudomonas aeruginosa*, on the other hand produces extracellular polysaccharides resembling alginic acid. The polymer consists mainly of D-mannuronic acid and varying amounts of L-guluronic acid.\(^{92-3}\) H.O. Bouveng et al.\(^{94}\) reported different extracellular polysaccharides from *P. aeruginosa*. These contain uronic acid, glucose, galactose, rhamnose, and mannose; and an aldobiuronic acid GlcUA-(1→3)-Man was isolated from a hydrolysate.

*P. prunicola* produces a levan consisting mainly of \(\beta\)-D-fructosyl-(2→6)-D-fructose together with \(\beta\)(2→1) branching linkages.\(^{95}\)

Eleven strains of *P. fluorescens* were studied by R.G. Eagon and R. Dedonder.\(^{96}\) They all produce extracellular polysaccharides consisting of glucose or galactose, with either mannose and/or glucosamine, fucose, rhamnose, xylose. Strain 11 polysaccharide contains glucose, glucosamine, and fucose (14:5:1). A disaccharide consisting of glucose and fucose was isolated from a hydrolysate as well as a tetrasaccharide consisting of fucose (2 molar proportions), glucose (1 molar proportion) and glucosamine (1 molar proportion).

*Agrobacterium* species,\(^{97-9}\) e.g. *A. tumefaciens* (virulent and non-virulent strains), *A. rhizogenes*, *A. radiobacter*, *A. rubi*, *A. pseudotsugae* produce extracellular polysaccharides (2-4 gm/L) which contain only glucose. They were shown to have a simple linear structure consisting mainly of \(\beta(1\rightarrow2)-D\)-glucose units. This type
of structure is not widely found outside the genus *Agrobacterium*.

*A. gypsophilae* produces only small amounts of polysaccharides. This contains galactose, glucose and uronic acid. The *Agrobacterium* polysaccharides, except that of *A. pseudotsugae*, all give non-viscous solutions in water.

The following table lists the components of extracellular polysaccharides, produced by 7 *Corynebacterium* species.

<table>
<thead>
<tr>
<th>Corynebacterium species</th>
<th>Components</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>insidiosum</em></td>
<td>gal:glc:fuc (3:2:4); pyruvic acid.</td>
</tr>
<tr>
<td><em>flaccumfacies</em></td>
<td>gal,glc,man,fuc (trace).</td>
</tr>
<tr>
<td><em>tritici</em></td>
<td>gal,glc,man.</td>
</tr>
<tr>
<td><em>rathayi</em></td>
<td>gal,glc,man.</td>
</tr>
<tr>
<td><em>fascians</em></td>
<td>gal,glc,man.</td>
</tr>
<tr>
<td><em>michiganense</em></td>
<td>gal,glc,man.</td>
</tr>
<tr>
<td><em>sepedonicum</em></td>
<td>gal,glc.</td>
</tr>
</tbody>
</table>

Oligosaccharides isolated from hydrolysates of the polysaccharide of *C. insidiosum* include

1. β-D-Glcp (1→4) L-Fuc,
2. β-D-Glcp (1→4) [D-Galp-(1→3)-L-Fuc],
3. C.Et.*Galp  \[\xrightarrow{\alpha} 3\] L-Fuc,
4. \[\xrightarrow{\beta} 4\] L-Fuc

* C.Et.Gal = 4,6-\_ (1-carboxyethylidene) D-Gal
B: Xanthomonas species and their extracellular polysaccharides

The genus Xanthomonas comprises species of microorganisms which are plant pathogens. It belongs to Pseudomonadaceae, a family of bacteria of the suborder Pseudomonadineae. Thirteen genera make up this family, typical examples of which are Pseudomonas, Rhizobium, Agrobacterium and Acetobacter. These microorganisms are Gram-positive and non-spore forming. The majority of the cells are rod-shaped to ovoid. Mobile species possess one or more flagella attached at the poles of the cells. They are never attached to the mucilaginous excretion, nor do they deposit iron or manganese in or on their cell walls, or capsules.

Species of Xanthomonas and Pseudomonas are closely related, although there are a few differences. X. species are monotrichous, possessing a single flagellum per cell whereas P. species have two flagella. X. species produce a yellow carotenoid pigment which is insoluble in the culture medium. It is mainly by virtue of this latter characteristic that species of Xanthomonas are differentiated from species of Pseudomonas. The former can oxidise a large variety of substances, but the products of their metabolism are not distinctive.

Xanthomonas species are among the five genera of bacterial plant pathogens, namely, Xanthomonas, Pseudomonas, Agrobacterium, Corynebacterium, and Erwinia. However, the latter two genera belong to the order, Eubacteriales. Together, these five genera comprise about 200 different species. Only Corynebacterium is Gram-positive. Unlike fungi, they do not form resting spores during their quiescent period. They remain dormant together with seeds, perennial plants, and plant residues. Their survival under natural or artificial environments present a great problem in the control of plant diseases.
The infections bacterial diseases of plants were noted as far back as 1891. The yellow disease of hyacinth was reported in 1895 to be due to a bacterium, now known as *X. hyacinthi*.

*Xanthomonas* species attack plants causing leaf, stem, and fruit spots, and occasionally blight of plants. The following list of *Xanthomonas* species and their host shows the extent and severity of these bacterial plant diseases.

<table>
<thead>
<tr>
<th><em>Xanthomonas</em> species</th>
<th>Ref.</th>
<th>Host (plants)</th>
</tr>
</thead>
<tbody>
<tr>
<td>alfalfa</td>
<td>106</td>
<td>alfalfa.</td>
</tr>
<tr>
<td>albineans</td>
<td>107</td>
<td>leaf scald of sugar cane.</td>
</tr>
<tr>
<td>campestris</td>
<td>108</td>
<td>black rot of cabbage, cauliflowers, brussel sprouts, collards, other crops of the same species.</td>
</tr>
<tr>
<td>citri</td>
<td>109</td>
<td>citrus canker affecting leaves, twigs and fruits.</td>
</tr>
<tr>
<td>incanae</td>
<td>110</td>
<td>stock of plants.</td>
</tr>
<tr>
<td>makatae</td>
<td>111</td>
<td>stem and leaves of the two makatae var. olitorii</td>
</tr>
<tr>
<td>malvacearum</td>
<td>112</td>
<td>natural Asiatic jute species.</td>
</tr>
<tr>
<td>oryzae</td>
<td>113</td>
<td>blight of rice.</td>
</tr>
<tr>
<td>oryzicola</td>
<td>114</td>
<td>leaf streak of rice.</td>
</tr>
<tr>
<td>phaseoli</td>
<td>115</td>
<td>bean, e.g. pustule of soya bean.</td>
</tr>
<tr>
<td>pruni</td>
<td>116</td>
<td>peaches, prunes, apricots, almonds, cherries, plums, nectarines.</td>
</tr>
<tr>
<td>stewartii</td>
<td>116</td>
<td>major disease of corn: severe wilt of sweet corn, and leaf blight of dent corn.</td>
</tr>
<tr>
<td>solanacearum</td>
<td>117</td>
<td>tobacco wilt.</td>
</tr>
<tr>
<td>vignicola</td>
<td>118</td>
<td>cowpea.</td>
</tr>
<tr>
<td>vesicatoria</td>
<td>119</td>
<td>bacterial spot of tomato</td>
</tr>
<tr>
<td>translucens, var.</td>
<td>120</td>
<td>pepper and</td>
</tr>
<tr>
<td>cerealis</td>
<td>121</td>
<td>wheat.</td>
</tr>
<tr>
<td>translucens, var.</td>
<td>122</td>
<td>black chaff of wheat.</td>
</tr>
<tr>
<td>translucens, var.</td>
<td>122</td>
<td>black chaff of wheat.</td>
</tr>
</tbody>
</table>
Other species include *X. beticola, X. hederae, X. juglandis*, *X. maculofoliigardeniae, X. papavericola, X. vasculorum, X. begonia*, and *X. pisi*. From the table, it can be seen that the specificity of the species is not always limited to one particular host. This would indicate a common mechanism for their pathogenic behaviour.

The pathogenicity and virulence of a particular species on the host plant depend on the specific strains that produce the disease. Most pathogenic *Xanthomonas* species produce an exudate at the site of lesion on the infected plants. Until about 17 years ago, most of the work carried out on these bacterial exudates was biological. It was found, for example, that although most phytopathogenic bacteria do not form spores, many are remarkably resistant to desiccation and survive for relatively long periods under dry conditions. It has been shown that the extracellular polysaccharides constituting the exudates produced by these bacteria protect the bacteria when dried or when exposed to ultraviolet light; and that the polysaccharides themselves are directly responsible for the disease. It is to be noted that the polysaccharides produced by these bacteria in culture are comparable to those produced as a result of infection in host plants. This protection offered by the exudate to the bacteria during desiccation may be due to the hydrophilic nature of the exudate. The hygroscopic properties of *X. phaseoli* exudate has been demonstrated and the bacterial cells were shown to survive in their exudate for as long as 125 days under a variety of conditions. The strong ultraviolet absorption of the exudate accounts for the protection of the bacterial cells against the lethal effect of the radiation. The toxicity of the polysaccharides to plants can be explained in terms of blocking of the
vascular systems, since most of the polysaccharides form viscous solutions. The virulence can be correlated to the amount of polysaccharide produced by the bacteria.

Several of these pathogenic bacteria also possess pectinolytic ability, and the pectic enzymes invading the vascular tissue have been investigated in the case of X. campestris. Furthermore, culture filtrates containing the pectinolytic enzymes caused disorganisation of tissue similar to that occurring in diseased plants.

The toxicity of the bacterial exopolysaccharide produced by X. campestris extends to a certain extent to rats and dogs. However the polymer has found considerable application especially as a thickening agent in food and in cosmetics, and as an improver in fibre production.

In 1958, V.G. Lilly et al. studied the laboratory scale production of polysaccharide by species of Xanthomonas. A suitable medium was found to consist of glucose in the form of starch, or sucrose, enzymatic casein hydrolysate, salts, if aerated and agitated. The ability of Xanthomonas species to utilise starch as a sole source of carbon differentiates this genus from Pseudomonas and Erwinia species.

X. phaseoli, X. campestris, X. malvacearum, X. carotae produced polysaccharide in good yield (≥ 6 gm/litre). X. transucens, X. hederae, X. papavericola, and X. vesicatoria were less productive. The strains of X. beticola and X. malvacearum used in another study did not produce appreciable amounts of polysaccharide.
X. stewartii, X. hyacinthi, X. maculofoliigardeniae, X. pruni, X. vignicola, also produced polysaccharides although figures for their yields were not quoted.

All of these extracellular polysaccharides examined, consist of glucuronic acid and glucose. In addition, some also contain mannose. The polysaccharides from X. stewartii and X. vesicatoria contain galactose instead of mannose. Polysaccharides from X. translucens f.sp. undulosa and X. papavericola contained also an additional unidentified sugar.

Only a few of these polysaccharides have been studied in detail. The extracellular polysaccharide produced by X. phaseoli was the first to be studied structurally by S.M. Lesley and R.M. Hochster. The purified polysaccharide (Cetavlon and Sevag's methods), shown to be homogeneous by examination in the analytical ultracentrifuge, by moving boundary electrophoresis and by gradient precipitation, was shown to contain equal quantities of glucuronic acid, glucose and mannose. No further structural details were given. This polysaccharide was the only one to be successfully examined on the ultracentrifuge, other workers have found it impossible to obtain any true boundary peak with any of these polysaccharides, and indeed that from X. phaseoli itself. The molecular weight average quoted for X. phaseoli extracellular polysaccharide is 19,500,000. Estimation of the molecular weight from the Schlieren pattern reported by S.M. Lesley and R.M. Hochster gave a much lower value.

In 1962, J.H. Sloneker et al reported on Xanthomonas campestris. This study was among the first detailed structural
accounts on these polysaccharides, and the first report of a bacterial polysaccharide containing pyruvic acid. The presence of both pyruvic acid and acetic acid residues provided steric factors to methylation and periodate oxidation, and posed several problems: for example, successive periodate oxidations and borohydride reductions were required to remove these acids and to cleave all units vulnerable to periodate attack.

The sugars and linkages reported in other polysaccharides of Xanthomonas species are tabulated below.

Table 1. Chemical composition of extracellular polysaccharides produced by Xanthomonas species.

<table>
<thead>
<tr>
<th>Species of Xanthomonas</th>
<th>Molar Proportion</th>
<th>Percentage</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>GlcUA</td>
<td>Glc</td>
<td>Gal</td>
</tr>
<tr>
<td>phaseoli</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>campestris</td>
<td>2.0</td>
<td>2.8</td>
<td>a</td>
</tr>
<tr>
<td>stewartii</td>
<td>1</td>
<td>4.5</td>
<td>3</td>
</tr>
<tr>
<td>oryzae</td>
<td>2</td>
<td>5</td>
<td>a</td>
</tr>
<tr>
<td>hyacinthi</td>
<td>+</td>
<td>1</td>
<td>a</td>
</tr>
<tr>
<td>translucens</td>
<td>+</td>
<td>1</td>
<td>a</td>
</tr>
<tr>
<td>maculofoliigardeniae</td>
<td>+</td>
<td>1</td>
<td>a</td>
</tr>
</tbody>
</table>

a = absent
+ = present, but proportion not determined
- = not investigated.
Table 2. Linkages found in the extracellular polysaccharides of *Xanthomonas* species.

<table>
<thead>
<tr>
<th>Xanthomonas species and method of linkage determination</th>
<th>GlcUA</th>
<th>Glc</th>
<th>Cal</th>
<th>Man</th>
</tr>
</thead>
<tbody>
<tr>
<td>campestris Periodate Oxidation</td>
<td>1,4-</td>
<td>1,4-</td>
<td>a</td>
<td>1,2-</td>
</tr>
<tr>
<td></td>
<td>1,4</td>
<td>1,2,4-</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>branching</td>
<td>1,3,4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>stewartii G.L.C. of methyl glycosides and periodate oxidation</td>
<td>+</td>
<td>1,6-</td>
<td>1,3-</td>
<td>a</td>
</tr>
<tr>
<td></td>
<td></td>
<td>end-group</td>
<td>1,6-</td>
<td>1,3,4,6-</td>
</tr>
<tr>
<td>oryzae crystalline derivatives of the methylated sugars and periodate oxidation</td>
<td>1,4-</td>
<td>1,4-</td>
<td>a</td>
<td>1,2-</td>
</tr>
<tr>
<td></td>
<td>end-group</td>
<td>1,3,4-</td>
<td>1,2,6-</td>
<td></td>
</tr>
<tr>
<td>coniophora, translucens, and maculofoliigardeniae. G.L.C. of the methyl glycosides.</td>
<td>+</td>
<td>1,4-</td>
<td>a</td>
<td>1,2-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1,3,4-</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>end-group</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a = absent

+ = present, but linkage not determined.
The reported molar proportion of the different sugars for the different polysaccharides must be looked at with some reservation, e.g., in the case of _X. phaseoli_, although a 1:1:1 ratio is reported no mention is made of the method of estimation. In the case of _X. hyacinthi, X. translucens_, and _X. maculofoliigardeniae_ no consideration is paid to the proportion of aldobiouronic acids left unhydrolysed. The proportion reported for the polysaccharide of _X. stewartii_ is only an estimate, whereas in the case of _X. campestris_ and _X. oryzae_ the proportion of the different sugar is checked against other results.

The pyruvic acid content of the extracellular polysaccharides from different _Xanthomonas_ species was determined by Orentas et al.\textsuperscript{149} It must be noted that this was determined by a non-specific method (colorimetrically as 2,4-dinitrophenyl hydrazone); and its presence and linkage have only been confirmed in the case of _X. campestris_.\textsuperscript{145} The presence of acetic acid was investigated only in the latter species. No crystalline derivative was prepared and its presence was shown only by thin layer chromatography of the hydroxamic acid derivative.

The following structural similarities are worth noting. The presence of the aldobiouronic acid, β-D-GlcUA-(1→2)-D-Man, has been reported in _X. campestris, X. oryzae_, and _X. hyacinthi_ extracellular polysaccharides.

Aldotriuronic acids have proved difficult to obtain in any quantity, although there is some evidence for the presence of the trisaccharide, β-D-GlcUA-(1→2)-Man-Glc, in the above 3 polysaccharides.

The tetrasaccharide, β-D-GlcUA-(1→2)-Man-Glc-Glc, has been tentatively identified in _X. campestris_ and _X. hyacinthi_ polysaccharides.
In contrast, \textit{X. stewartii} extracellular polysaccharide which contains galactose instead of mannose gave the aldobiuronic acid, \(\beta\)-GlcUA\((1\rightarrow 4)\) D-Cal.

Among the neutral disaccharides reported are

<table>
<thead>
<tr>
<th></th>
<th>(\beta)-D-Glc((1\rightarrow 4)) D-Man</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{X. hyacinthi}</td>
<td>(\beta)-D-Glc((1\rightarrow 4)) D-Man</td>
</tr>
<tr>
<td></td>
<td>(\beta)-D-Glc((1\rightarrow 4)) D-Glc</td>
</tr>
<tr>
<td>\textit{X. campestris}</td>
<td>(\beta)-D-Glc((1\rightarrow 4)) D-Glc</td>
</tr>
<tr>
<td>\textit{X. stewartii}</td>
<td>(\beta)-D-Glc((1\rightarrow 3)) D-Gal</td>
</tr>
<tr>
<td></td>
<td>(\beta)-D-Glc((1\rightarrow 6)) D-Gal</td>
</tr>
<tr>
<td></td>
<td>(\beta)-D-Gal((1\rightarrow 3)) D-Gal</td>
</tr>
</tbody>
</table>

The work reported in this thesis is of a structural investigation of extracellular mucilage of a bacterium characterised as being of the genus \textit{Xanthomonas} and designated by us S19.
GENERAL METHODS

GM.1 Common procedures

(a) All evaporations were carried out under reduced pressure at 40°.

(b) Specific rotations were measured in a 1 dm polarimeter microcell (volume = ca. 1 ml) in a Perkin Elmer 141 polarimeter, using the sodium-D-line. Unless otherwise stated, water was used as solvent.

(c) Dialysis was carried out in Visking cellophane tubing against water with toluene added as bacteriostat.

(d) Melting points were determined on a Hoover capillary or Gallenkamp micro-melting point apparatus.

(e) Water used was deionised.

(f) Freeze-drying: samples were first frozen in a cardice-acetone mixture before being placed on the freeze-drier.

(g) All solutions and hydrolysates were filtered through millipore filters (0.45 μ) before quantitative determinations.

(h) Ash content was determined after ashing in a platinum crucible.
GM.2 Assays and Analyses

(a) Determination of carbohydrate content

This was by the phenol-sulphuric acid method. Standard graphs of monosaccharides or appropriate mixtures of monosaccharides were made as follows:— About 10 mg of dry carbohydrate accurately weighed were dissolved in water (100 ml). Aliquots (0-1 ml) were taken and made up to 1 ml with water to give 10-100 \( \mu g/ml \) of carbohydrate. One millilitre of 4\% phenol in water was added, followed by 5 ml of analar concentrated sulphuric acid quickly from an automatic dispenser. After mixing and cooling to room temperature, the absorption was measured in a spectrophotometer (Unicam SP 500 or EEL 197) at 487 nm. The absorptions were plotted against concentration to give a straight line graph, from which the carbohydrate content of unknown was read.

(b) Uronic acid estimation

(i) Naphthoresorcinol test

The sample (40 mg) was boiled for 1 min with conc. HCl and a few crystals of naphthoresorcinol. The dark blue colour was extracted into diethyl ether after cooling.

(ii) Modified carbazole method. Reagents:

(1) 0.025M sodium tetraborate decahydrate (AR, Borax) in sulphuric acid sp. gr. 1.84 (AR).

(2) 0.125\% carbazole in absolute methanol (AR). This is stable for 12 weeks at 4\(^{\circ}\)C in the dark.

(3) From a stock solution of dried glucuronolactone or glucuronic acid, standard solutions containing 4-40 \( \mu g/ml \) were prepared by dilution with deionised water saturated with benzoic acid. The stock solution is stable for 6 months at 4\(^{\circ}\).
Procedure: Reagent (1) (5 ml) was placed in Quickfit tubes and cooled to 4°C. The sample or standard (1 ml) was carefully layered on top of the acid. The tubes were closed and the rack shaken at first gently, then vigorously with constant cooling. At no time should the temperature of the mixture exceed room temperature.

The tubes were then heated at 100°C for 10 min and quickly cooled to room temperature. Carbazole reagent (2) (0.2 ml) was added, the tubes were shaken again, heated at 100°C for 15 min and again quickly cooled to room temperature.

The optical density (OD) was then read at 530 nm on a Unicam SP 500. The OD of the blank against sulphuric acid should be below 0.025. Standard graphs for different acids were prepared.

(iii) **Using metahydroxydiphenyl**

N. Blumenkrantz and G. Asboe-Hansen method\textsuperscript{154} was used.

Reagents:

1. Metahydroxydiphenyl: 0.15% in 0.5% NaOH. The reagent was kept at 4°C in the dark; stable for 5 weeks.

2. Acid/tetraborate solution: 0.0125M solution of sodium tetraborate decahydrate in concentrated sulphuric acid, Sp. gr. 1.84 (AR).

Procedure: To the acid/tetraborate solution (5 ml) in Quickfit tubes cooled to 4°C, the sample (1 ml) containing 10-60 μg of uronic acid in water was added. After mixing, and allowing the solution to reach room temperature, the tubes were heated at 100°C for 5 min. After cooling to room temperature again, the metahydroxydiphenyl reagent (0.1 ml) was added. The colour was developed by vigorous mixing and then read within 5 min at 520 nm.
Standard glucuronic acid and glucurone did not give any colour after boiling with the acid reagent. Therefore no blank reading was subtracted. However, unknown sample blanks were carried out by using 0.5% NaOH (0.1 ml) instead of the meta-hydroxydiphenyl reagent. This reading was subtracted from the total absorbance. The uronic acid content was read off standard graphs.

(c) Glucose content

The Boehringer blood sugar kit was used. To the sample (0.1 ml) at room temperature, the enzyme solution (5 ml) was added. The solution was mixed and kept away from direct sunlight. After 30 min. the optical density was measured at 436 nm. A calibration graph was plotted by using the standard sample (0.1 ml) containing 5-50 \( \mu \)g of glucose. A calibration graph was plotted each time a determination was made.

(d) Galactose content

Galactose was determined using a Galactostat kit available from Worthington Biochemical Corporation whose procedure was followed.

(e) Sulphate content

The polysaccharide (10 mg) was digested in a sealed tube with analar HNO\(_3\) (s.g. = 1.42) (1 ml, plus a few mg of NaCl) for 12 h at 100\(^\circ\). The solution was evaporated to dryness and the residual solid treated with concentrated HCl (ca. 0.5 ml), and the mixture evaporated to dryness again. The tube was placed in an oven at 105\(^\circ\) for 2 h. The sample was then ready for sulphate determination.

The following modification of the Jones and Letham\(^{155}\) method was used.

Dry potassium sulphate (0.3625 g) was dissolved in water (1 L) to give a standard solution containing 200 mg of sulphate/litre.
To the sulphate solutions (0.5 ml containing 30-100 μg of sulphate) in micro centrifuge tubes, were added the reagent, 4-chloro-4'-amino-diphenyl (0.5 ml of 0.19% in 0.1N HCl) and a trace of solid hexadecyltrimethylammonium bromide. After mixing, the solutions, including a blank sample, were kept for 2 hours, and then centrifuged. Aliquots (0.2 ml) of the supernatant were removed and diluted to 25 ml with 0.1N-HCl. The optical densities were read at 254 nm. The differences over the blank reading were plotted against the known concentrations to give a standard graph from which unknown concentrations were read. An ammonium molybdate test was usually carried out to detect any phosphate which, if present, would interfere.

(f) Acetate content

Acetate was determined by the Hestrin method. Reagents: (1) Hydroxylamine: Hydroxylamine hydrochloride, 2M (13.9 g/100 ml). The solution should be stored in the cold.

(2) Alkali: 3.5N-sodium hydroxide (14 g/100 ml).

(3) Acid: conc. HCl, sp.gr. 1.18, diluted with 2 parts by volume of water.

(4) Iron: 0.37M-ferric chloride, in 0.1N-HCl i.e. (10 g/100 ml).

(5) Standard solution: Glucose pentaacetate (39.9 g/100 ml) in 0.01N-sodium acetate buffer (pH 4.5). This stock solution may be kept in the cold for at least a fortnight without measurable loss. Standard solutions (0-5 μ moles of acetate/ml) were made by suitable dilution of the stock solution (containing 5 μ moles of acetate/ml) with the sodium acetate buffer. Unknowns were also made up in the same buffer.

Procedure: Equal volumes of reagent (1) and (2) were mixed freshly before use to make an alkaline hydroxylamine reagent. The mixture keeps
for about 3 h. at room temperature. This reagent (2 ml) was added to
the sample (1 ml). After at least 1 minute, or longer if desired, the
pH was brought to 1.2 ± 0.2 with 1.0 ml of acid, and 1.0 ml of the iron
solution was added. The optical densities of the solutions were
promptly read at 454 nm on EEL 197. Formation of gas bubbles in the
cell was avoided by adequate mixing after the addition of each solution.

Correction for non-specific colour was made by repeating the
procedure as described, except that the order of addition of hydroxylamine,
alkali, and acid was reversed. With this order of addition esters do
not form any hydroxamic acid derivatives.

The optical densities were plotted against the known concentrations
to give a standard calibration graph from which acetate concentrations
for unknowns were read.

(g) Degree of polymerisation of oligosaccharides

This was determined according to the Timell modification157
of the method of Peat et al.158 Three solutions were prepared as follows:

1. A blank containing water (0.5 ml) and 2% potassium
borohydride solution (0.5 ml).

2. An aqueous sugar solution (0.5 ml, containing 60-80 μg
as monosaccharide) and 2% potassium borohydride (0.5 ml).

3. A 2N-sulphuric acid solution (0.5 ml) containing the same
amount of sugar as in solution(2) and 2% potassium borohydride solution
(0.5 ml).

These mixtures were left at RT for 6-20 h. Then, phenol-sulphuric
acid determination (CM.2a) was carried out on each solution. The
optical density of solution 1 was used as blank.

The DP of the carbohydrate substance is given by the relationship:

\[
\text{DP} = \frac{(O.D)N}{(O.D)N - (O.D)R}, \quad \text{where}
\]

\((O.D)N\) = optical density of non-reduced solution 3.

\((O.D)R\) = optical density of reduced solution 2.
This equation applies only for homosaccharides. Otherwise, the O.D. may be converted into the corresponding weight of sugar (read off the appropriate standard graph). Then

\[
D = \frac{\text{wt. of non-reduced carbohydrate}}{\text{wt. of non-reduced carbohydrate} - \text{wt. of reduced carbohydrate}}
\]

(h) **Pyrubic acid estimation**\(^{159,160}\)

(1) Triethanolamine, \(N(CH_2CH_2OH)_3 = [\text{TEA}]\).
Aqueous 0.1M-TEA buffer was made immediately before use by diluting a stock 1.0M-TEA solution (13.26 ml in 100 ml water). The pH was adjusted to 7.6 with a few drops of 5N-HCl.

(2) 1% Nicotinamide adenine dinucleotide (reduced form) (NADH) in 0.1% NaHCO\(_3\) solution.

(3) Lactic acid dehydrogenase (LDH) (Sigma Type II from rabbit muscle). From the quoted concentration of enzyme, suitable dilution was calculated to give 18 units of enzyme per 50μl of solution.

(4) Standard pyruvic acid solution. Pyruvic acid was distilled at 36-38°C under reduced pressure (0.1 - 0.3 mm) to give a clear colourless liquid. This was kept at 0°C and was used to make standard aqueous solutions (0 - 3 mg/100 ml).

Procedure: To buffer (2 ml), standard pyruvic acid solutions or sample (2 ml), NADH (50μl) was added and mixed carefully. The optical density was read at 340 nm. This reading is \(A_0\). Then LDH (50μl) was added and the solutions mixed. Readings were taken after 5 min and 10 min, after which the optical density was usually constant. This reading is \(A_f\). A sealed capillary tube was found very convenient for mixing without loss of solvent.

\[(A_0 V_0 - A_f V_f)\] was calculated where \(V_0 = 4.05\) and \(V_f = 4.10\).

This, called \(\Delta AV\), was plotted against the concentration of pyruvic acid to give a straight line graph.
(i) **Protein analysis and estimation**

For amino acid analysis any protein was hydrolysed with 1?N-HCl for 6 h in a sealed tube. The hydrolysate was evaporated to dryness.

Nitrogen content was measured by A. Bernhardt (West Germany) and the protein content calculated by multiplying by 6.25.

(j) **Thiobarbituric acid test (TBA)**

The TBA test for the presence of 4,5-unsaturated acid was used with the following modifications. The sample in 0.20 ml or less of solution was added to 0.025N-HIO₄ in 0.125N-H₂SO₄ (0.25 ml). After 20 min. at room temperature, 2% sodium arsenite in 0.5N-HCl (0.5 ml) was added with shaking and the solution was permitted to stand for 2 minutes.

0.3% TBA (2 ml) (pH 2) was added and after stirring, the mixture was heated at 100°C for 10 min. When the mixture had cooled, its ultra-violet spectrum was measured on a Unicam SP 500 to detect any absorption maximum at 548 nm.
GM.3 Acid Hydrolyses

(a) with formic acid

The sample (1-25 mg) was hydrolysed with 90% formic acid (1 ml) in a Quickfit tube (sealed with a piece of cut rubber tubing and copper wire) at 100° in an atmosphere of carbon dioxide for 6 h. Then the cooled tube was opened, water (5 vol) was added and the hydrolysate heated on the boiling water bath for a further 2 h. The mixture was evaporated down to dryness together with methanol to remove formic acid.

(b) with sulphuric acid

(i) The sample (1-25 mg) was hydrolysed with N-sulphuric acid (2 ml) under an atmosphere of carbon dioxide at 100° for 4 h. After cooling and dilution with water, the hydrolysate was neutralised with solid A.R. barium carbonate. Completion of neutralisation took a few hours because of the slow precipitation of barium sulphate. The neutralisate was filtered through washed Whatman No.5 fine filter paper; the residual salts were washed 2 or 3 times with cold water. The filtrate and washings were evaporated to dryness.

(ii) When a larger amount of sulphuric acid was used, the hydrolysate was carefully brought to pH 4-5 with a saturated solution of barium hydroxide and then further neutralised as above.

(iii) When the sample was hydrolysed with 0.5 ml or less of N-acid, the hydrolysate was neutralised by shaking with N,N-dioctylmethylamine (C_{17}H_{35}N) (5% v/v in chloroform) (twice) and the neutralysate freed from residual amine by shaking with chloroform.

(c) with oxalic acid

The sample was hydrolysed with oxalic acid (0.5N → 5N) at 70-100° as required and was neutralised with solid calcium carbonate. The hydrolysate was worked up as in [GM.3 (b) (i)].
GM.4 General Reactions and Preparations

(a) Preparation of methanolic hydrochloride

Absolute methanol was distilled off after treatment of commercial methanol (AR) with magnesium activated by iodine. The dry methanol was stored over dry molecular sieve type 3Å. Dry hydrogen chloride gas was passed into dry methanol until saturation was reached. An aliquot was titrated with N-NaOH and diluted with dry methanol as required.

In an alternative method of preparation, acetyl chloride (5 ml) was added to methanol (95 ml). This gave a 3% methanolic hydrogen chloride solution.

(b) Methanolysis

The dry sample was refluxed with ca. 7% methanolic hydrogen chloride (1-5 ml) for 6 - 18 h. A calcium chloride tube was used to keep a dry atmosphere. The methanolysate was neutralised with dry silver carbonate, filtered, and the precipitate washed with dry methanol. The combined filtrate and washings were evaporated to dryness.

(c) Reduction with sodium borohydride

The sample (25 mg) was dissolved in water (3 ml) or water/methanol (1:1 v/v), and a small spatula tip of sodium borohydride was added to give approximately a 2% borohydride solution. It was left standing for about 6 h. If the solution was not still alkaline by this stage, more sodium borohydride must be added and the mixture left for a further 10 h, and then neutralised with IR 120 (H+) resin. After filtration, the filtrate was co-distilled with methanol to remove boric acid, and finally evaporated to dryness; complete reduction was checked with Fehling's solution.
(d) Conversion into methylglycosides

The dry sample, usually free sugars or methylated sugars, was refluxed in dry methanol with methanol treated Amberlite IR 120 (H\(^+\)) resin as catalyst for 3 - 18 h. A calcium chloride tube must be used to keep a dry atmosphere. Recommended ratios of materials are, for example,

\[
\text{D-glucose : resin} = 2-3 : 1 \text{ w/w} \\
\text{Methanol : glucose} = 8-45: 1 \text{ mole/mole}
\]

The methylglycosides for GLC were separated from the resin with a Pasteur pipette and evaporated to a dry syrup.

(e) Preparation of trimethylsilyl derivatives

(i) The dry product (e.g. sugars) was dissolved in dry pyridine (0.5 ml), and hexamethyldisilazane (0.2 ml) and trichloromethyl-silazane (0.1 ml) were added. The stoppered flask was shaken for 10 mins. Then the mixture was centrifuged, the supernatant liquid taken down to dryness and the derived solids dissolved in dry n-hexane for gas-liquid chromatographic analysis.

(ii) To the dry sample, dry pyridine (1.0 ml), hexamethyldisilazane (0.9 ml) and trifluoroacetic acid (99%, 0.1 ml) were added successively. The stoppered flask was shaken vigorously for 30 sec, and allowed to stand for 30 min. with occasional shaking and with intermittent release of gas pressure. Properly prepared derivatives should be clear. The sample was then ready for injection into the gas chromatograph.
(f) Acetylation

Sugars or their derivatives (dried syrups) were acetylated by dissolving them in acetic anhydride-pyridine mixture (1:1 v/v, 2 ml) and heating the mixture under anhydrous conditions (conveniently in a sealed tube) for 15 min at 100°. The acetates after evaporation to dryness and dissolution in dry chloroform were ready for GLC analysis. To avoid extraneous peaks, the chloroform solution was washed twice with N-HCl followed by aqueous NaHCO₃ solution (fast) and water. The solution dried over CaCl₂ overnight was concentrated to a small volume for injection.

(g) Reduction of uronic acid to the neutral sugar

The material was first converted into the methylester methylglycoside [GM. 4 (d)] and then reduced with sodium borohydride [GM. 4 (c)] to the neutral methylglycoside. This was hydrolysed with sulphuric acid [GM. 3 (b)(i)] or with 90% formic acid [GM 3 (a)] to yield the free sugar.

(h) Modified Hakomori methylation

The Sandford and Conrad modification of Hakomori methylation was used.

(i) Preparation of dry dimethylsulphoxide

Dimethylsulphoxide (DMSO) (b.p. = 189°C, 85-92°C) was distilled over calcium hydride. The dry distillate was stored over molecular sieve, type 4Å. (The sieve was previously dried overnight at 140°C in an oven).
(ii) **Preparation of dry methyl iodide**

This reagent is carcinogenic. Methyl iodide was distilled over silver oxide (previously dried at 105°C in a drying pistol) and stored at 4°C in a dark bottle over silver oxide.

(iii) **Preparation of dimethyl sulphinyl carbanion**

Sodium hydride (0.75 g) (i.e. 1.5 g of 50% coated with mineral oil) was weighed into a 250 ml three-necked flask, and was washed with n-pentane (five times 50 - 100 ml portions), the latter being decanted off after each washing. (The decantate may contain a small amount of sodium hydride and can be rendered safe by the slow addition of ethyl alcohol). The pentane was previously dried by storing over sodium wire usually for about 1 week.

After the final washing, the flask was fitted with an air condenser, a fine bleed and a thermometer reaching near the bottom of the flask. The apparatus was evacuated through the bleed while the sodium hydride was stirred over a magnetic stirrer (teflon follower). Dry nitrogen (through CaCl₂ tower) was flushed through the bleed into the flask which was then evacuated again. The process was repeated several times (6-7) so as to provide a nitrogen atmosphere. By this time, the sodium hydride must be dry; otherwise the system was evacuated for about 30 min. more.

Nitrogen was allowed to flush through continuously and dry DMSO (15 ml) was added by means of a syringe to the flask from the top of the condenser. A vigorous reaction took place with the evolution of hydrogen. The flask was heated in an oil bath at 45 - 55°C. for 1 - 2 h until the reaction mixture became clear (light green) and hydrogen evolution ceased. The carbanion was stored under nitrogen in
McCartney bottles. It can be kept at 0° but decays in strength rapidly at room temperature. It must be titrated (1 ml): (N/10 HCl) immediately before use and should be about 2N.

(iv) Methylation procedure

The dry sample (5 – 30 mg) was dissolved or swelled in dry DMSO (1 – 2 ml) under nitrogen in a McCartney bottle. This often took 24 h or more. The 2M-carbanion (1 ml) was added by means of a syringe through the rubber cap, and the mixture usually became gelatinous. The bottle was agitated in an ultrasonic bath for 1 h. (KS 100, 50 c/s), and then for 6 h on a mechanical shaker. Methyl iodide (0.1 ml) was added while the bottle was cooled in water, followed by agitation in the ultrasonic bath for 20 mins. A second portion of carbanion (1 ml) was added and the process repeated using 1 ml of methyl iodide this time. The reaction mixture was poured into water (25 ml) and dialysed for about 2 days, when a reddish oily layer became solid.

The water was evaporated off. The methylated product was acid hydrolysed and analysed as the methylated methylglycosides and methylated alditol acetates. 174

(i) Modified Kuhn methylation

The C.T. Bishop and O. Perila modification 175 of the Kuhn 176 methylation was used.

The oligosaccharide (0.5 – 2 mg) was shaken with methyl iodide (0.2 ml), N,N-dimethylformamide (0.2 ml) and dry silver oxide (0.2 g) at room temperature for 18 h in the dark. The reaction was begun at 0°C and maintained at that temperature for 30 min. The mixture was filtered, the residue washed with dry chloroform and the combined
filtrate and washings were dried with anhydrous copper sulphate. The methylated product was obtained after evaporation to dryness under reduced pressure (0.03 mm) at room temperature.

(j) Demethylation

The sugar sample in dry dichloromethane (2 ml) was cooled to -30°. Boron trichloride (1 - 2 g) cooled to -30° was added and the mixture kept at -30° under anhydrous conditions for 30 min. The solution was then allowed to warm up to room temperature and allowed to stand under anhydrous conditions for 16 h. The remaining solvent was removed under vacuum, and methanol (3 x 3 ml) was added and evaporated. The syrup was examined chromatographically.

(k) Separation of neutral and acid sugars

This method uses an anion exchange resin column of Deacidite FF-IP (Permutit SRA 67, 200 - 400 mesh). The resin was provided in the chloride form and must be washed with sodium hydroxide (2N) until chloride free (silver nitrate test), then with deionised water until neutral (pH 7), and with formic acid (2N, about 5 - 10 litres) and finally with deionised water to a constant pH (ca. 6.5). The column was then ready for use.

After loading the sample (which must be in the free acid form), neutral sugars were eluted with water and acid sugars were eluted with (0 - 2N)-formic acid. Care must be taken to remove all neutral sugars, especially oligosaccharides so that these do not elute with the acid fraction.
(1) Periodate oxidation

The polysaccharide (500 mg in water, 500 ml) was oxidised with 0.03M-sodium metaperiodate (500 ml) in the dark at room temperature for 2 - 15 days. Ethylene glycol (1 ml) was added to destroy excess periodate; the reaction was monitored by the following methods (i) spectrophotometric method; (ii) iodometric titration; (iii) arsenite method, and (iv) spectrometric determination of iodate after treatment with resin.

(m) Reduction of polyaldehyde to polyalcohol

(i) Without buffer

The polyaldehyde (from 500 mg of polysaccharide) solution was reduced with sodium borohydride (1 g). The solution was stirred for 2 h at room temperature and then left at 2° for 20 h. It was dialysed (3 - 4 days) and freeze-dried to give the polyalcohol. An aliquot of the polyol solution was tested for reducing power.

(ii) With buffer

The polyaldehyde (from 500 mg of polysaccharide) solution was made 0.05M with respect to boric acid and cooled to 0°. Sodium borohydride (1 g) in water was added dropwise with stirring at 0°. The solution (pH 9) was then allowed to stand overnight at 2°. The polyalcohol was recovered after extensive dialysis (3 - 4 days) and freeze-drying. Test for reducing power was carried out on an aliquot of the polyol solution.

(n) Reduction with lithium aluminium hydride

The methylated polysaccharide (60 mg) was suspended in dry tetrahydrofuran (THF) (100 ml). Lithium aluminium hydride (60 mg in THF, 5 ml) was added gradually to the suspension at room temperature with occasional shaking. After half an hour the mixture was refluxed for 2 h (b.p. of THF = 66°C).
Further LiAlH$_4$ (40 mg in THF, 5 ml) was added and the mixture refluxed for 0.5 h. After cooling, excess LiAlH$_4$ was destroyed by the addition of water, and the solution brought to pH 4 with dilute H$_2$SO$_4$. After concentration, the reduced product was extracted into chloroform. The extract was evaporated to a clear syrup.

**CM.5 Chromatography**

(a) Paper chromatography

Whatman No. 1 paper was used for qualitative paper chromatography. The following solvent systems were used for descending chromatography:

(i) ethyl acetate : acetic acid : formic acid : water (18:3:1:4).
(ii) n-butanol : pyridine : water (6:4:3).
(iii) trough = ethyl acetate : pyridine : acetic acid : water (5:5:1:3).  
(iv) n-butanol : ethanol : water (40:11:19).  
(v) ethylmethylketone : acetic acid : water (9:1:1) saturated with boric acid.
(vi) Tate and Lyle solvent.
(vii) acetone : n-butanol : water (5:3:2).
(viii) ethylacetate : acetic acid : water (6:3:2) (freshly prepared).
(ix) water saturated ethylmethylketone + 3% w/v cetylpyridinium chloride.

The latter two solvents are used for sugar sulphates.

(x) n-butanol : acetic acid : water (4:1:5).  
    (upper layer in trough, lower layer in tank).
(b) Development of chromatograms

Qualitative paper chromatograms were usually dried in an oven at 105°. It must be noted that papers eluted with borate were first washed in glacial acetic acid, and cetylpyridinium chloride papers in chloroform before the following staining techniques were used.

(i) Silver nitrate dip. 187

The paper was sequentially dipped through silver nitrate, sodium hydroxide and sodium thiosulphate solutions with drying at room temperature between each solution.

AgNO₃ : Stock solution = saturated aqueous silver nitrate. An aliquot (5 ml) was diluted with acetone (1 L) and a few drops of water added to redissolve the white precipitate.

NaOH : Sodium hydroxide (20 g) in water (40 ml) was diluted with ethanol (960 ml).

Thiosulphate: 10% w/v of aqueous sodium thiosulphate.

(ii) Aniline oxalate spray 188 for reducing sugars.

The paper was sprayed with aniline oxalate (25 g) in 50% aqueous ethanol (1 L) and quickly placed in an oven at 105°.

(iii) Urea hydrochloride spray for Ketoses.

The reagent consists of urea (10 g), conc. HCl (8 ml), water (32 ml) and ethanol (200 ml). The blue colour specific for ketoses developed after 5 min at 100°C.

(iv) Triketohydrindene (Ninhydrin) spray for amino acids.

A freshly made 2% solution of triketohydrindene in alcohol was used. The colour developed after 5 min at 70°C.
(v) **Glucose oxidase dip for D-glucose.**

The glucostat and chromogen supplied by Worthington Biochemical Company was used. Each reagent was separately dissolved in water (50 ml), and kept at 4°C in the dark. The paper was dipped through each solution, with drying at room temperature in between. Exposure to direct sunlight was avoided. A pink colour developed in less than 1 min at room temperature.

(vi) **Galactose oxidase dip** for D-galactose.

The reagent consists of galactose oxidase (2 - 3 mg), horse radish peroxidase (2 - 3 mg), o-dianisidine (10 mg), in 0.1M-phosphate buffer, pH 6.9 (10 ml). The colour develops at room temperature within 10 - 15 min.

(vii) **Tetrazolium spray** for glycosidic links.

The spray was a freshly prepared mixture of 2% aqueous or methanolic triphenyltetrazolium hydrochloride and N-NaOH (1:1). After spraying the paper was heated at 40°C on a steam bath for 10 mins. Intense red colour indicates the presence of non-1,2-linkages. 2-O-methylfucose was used as control.

(viii) **Aniline-diphenylamine spray** for glycosidic links.

Solution 1: aniline (4 ml) in acetone (100 ml).

Solution 2: diphenylamine (4 g) in acetone (100 ml).

Solutions 1 and 2 were mixed together with 85% orthophosphoric acid (20 ml) and kept at 0°C in the dark. After spraying, the paper was heated at 80°C for 2 - 3 mins. Intense blue colour indicates 1,4-linkages and greyish-green colour indicates 1,3-linkages.

(ix) **Bromophenol blue spray for bases and acids.**

A 0.001% w/v solution in ethanol was used. The pH was brought to pH 4.6 with alkali.
(c) **Preparative paper chromatography**

Whatman 3 MM or No. 17\(^{193}\) paper was used. These were previously washed with water for 24 h, and dried at room temperature. Usual load of sugars was about 0.1 g for 3 MM paper and 0.2 - 0.3 g for No. 17 paper. Strips were eluted with water until carbohydrate-free.

(d) **Paper electrophoresis**

The Shandon high voltage electrophoresis apparatus model L24 was used with Whatman 3 MM paper and the following electrolytes.

(i) **Molybdate buffer\(^{194}\)**

Sodium molybdate dihydrate (25 g) in water (1200 ml) was brought to pH 5.0 with conc. \(\text{H}_2\text{SO}_4\). The voltage applied was 30 - 60 v/cm for 1 - 3 h. The non-migrating marker was glucose.

(ii) **Borate buffer\(^{194}\)**

Sodium borate (0.2 M) in water was adjusted to pH 10 with NaOH. The voltage applied was 30 - 60 volts/cm for 1 - 2 h.

(iii) **Borate buffer with calcium ions\(^{195}\)**

Aqueous 0.01M-sodium borate solution \((\text{Na}_2\text{B}_4\text{O}_7\cdot10\text{H}_2\text{O})\) was made 0.005M with respect to \(\text{CaCl}_2\). The pH was adjusted to 9.2. Electrophoresis was run at 0.5 mA/cm for 2 h.

(iv) **Pyridine-acetic acid buffer**

Whatman No. 1 paper was often used for this buffer which consists of 0.1M-acetic acid (11.44 ml of glacial acetic acid in 1988.6 ml of water) which was brought to pH 6.0 - 6.5 with pyridine (ca. 120 ml). The voltage applied was 3000 volts for 1 h, giving about 30 - 40 mA for No. 1 paper and about 70 - 90 mA for 3 MM paper. Glucose was the non-migrating marker.
(e) **Thin layer chromatography (TLC)**

Precoated Kodak cellulose or polygram Sil G plates were used in solvents

(i) ethyl acetate

(ii) water saturated phenol

(iii) water saturated n-butanol


(f) **Gas-liquid chromatography (GLC)**

(i) A Pye Argon gas chromatograph with Argon ionisation detector and argon as carrier gas was used. Glass columns (1m x 5 mm) were packed with (a) 15% Butane-1,4-diolsuccinate polyester on silane treated Celite (average temperature used = 170°C).

(β) 10% Polyphenyl ether[m-bis (m-phenoxyphenoxy)- benzene] on silane treated Celite (average temperature used = 165°C).

(γ) 7.5% Apiezon K on silane treated chromosorb W. (average temperature = 175°C).

(ii) A Pye 104 gas chromatograph with flame ionisation detector and nitrogen as carrier gas was used. Glass columns (3m x 5mm) were packed with

(a) 3% OV 225 on Gaschrom Q (average temperature used = 175°C).

(β) 20% Carbowax 1500 on chromosorb P (average temperature used = 50°C).

(γ) 3% OV 17 on chrom W (AW-DMCS).

Retention times (T) of (a) methylated methylglycosides (b) methylated alditol acetate and (c) TMS-derivatives were measured relative to

(a) 2,3,4,6-tetra-O-methyl-β-methylglucoside (TMG) (b) 1,5-di-O-acetyl-2,3,4,6-tetra-O-methyl glucitol (TMGA) and (c) xylitol-TMS derivative respectively.
(g) Gas-liquid chromatography linked to mass spectrometry (GC-MS).

A Perkin-Elmer F11 gas chromatograph with flame ionisation detector and helium as carrier gas was coupled via an all-glass system through a Watson-Biemann separator to a Hitachi RMS-4 mass spectrometer. Mass spectra were obtained by operating the ion source at 200°C, 50 eV, and 80 μA target current. Glass columns used were

(i) 4 m x 1.6 mm, packed with 3% OV 225 on Gaschrom Q. (average temperature = 200°C).

(ii) 2 m x 1.6 mm, packed with 3% OV 17 on Chrom W (AW-DMCS; 85-100 m.u.) (average temperature = 220°C).

(h) Spectrometry

(i) Ultra-violet and visible spectroscopy

Quartz cells (4 ml capacity) were used. Spectrophotometers were either the Perkin-Elmer model 137 or the Unicam SP500 (series 2 ultra-violet and visible) or the EEL 197 (Evans Electroselenium Ltd.).

(ii) Infra-red spectroscopy

Polysaccharide films or KBr discs were made. The Perkin-Elmer model 337 grating or the Unicam SP1000 or the Perkin-Elmer model 325 infra-red spectrophotometer was used.
EXPERIMENTAL

Expt. 1. Examination of the crude polysaccharide

The crude polysaccharide (ca. 25 mg) was hydrolysed with 90% formic acid [GM 3a] and N-sulphuric acid [GM 3(b)i], and the hydrolysates were analysed by chromatography (solvents [GM 5(a)i, ii, iv] and sprays [GM 5(b)i, ii]).

Expt. 2. Purification of the polysaccharide

The crude polysaccharide (22 g) after shaking in water (4 l) to give a thick gel was re-precipitated with ethanol (5-6 vol). The precipitate was recovered on silk cloth, dissolved in water and extensively dialysed. The dialysis sac content was freeze-dried (16 g).

Expt. 3. Examination of the purified polysaccharide

(a) Carbohydrate content

The polysaccharide (10 mg) was dissolved in water (350 ml) by shaking for 24 h. The carbohydrate content of the solution was then determined by the phenol-sulphuric acid method [CM 2a]. Calculation was based on a standard glucose graph.

(b) Carbohydrate content of various hydrolysates

Weighed aliquots of the polysaccharide were each hydrolysed with 0.5M-H$_2$SO$_4$ (in triplicate) [GM 3(b) i], with 0.25M-H$_2$SO$_4$ and with 90% formic acid [GM 3a]. In each case, the carbohydrate content was determined on the hydrolysate and the percentage recovery calculated.

(c) Examination of the polysaccharide hydrolysates

The syrups from the 0.5M-sulphuric acid and 90% formic acid hydrolysies were examined as follows.
(i) Paper chromatography: Chromatograms (solvents [CM 5(a) i-iv]) were developed with silver nitrate, aniline oxalate, glucose oxidase, and galactose oxidase (sprays [CM 5(b) i, ii, v, vi] respectively).

(ii) Ionophoresis: This was carried out in borate buffer (pH 10) and pyridine/acetic acid buffer (pH 6.5) [CM 5 (d) iii, iv].

(iii) Gas-Liquid chromatography was carried out on the sugar-TMS, alditol-TMS, and alditol-acetate derivatives both before and after reduction of the uronic acid [CM 5(f) (i) 7; CM 5(f) (ii) a], and [CM 4(c,d,f)].

(d) Characterisation of the constituent sugars

The polysaccharide (1.13 g, 789 mg carbohydrate) was partially hydrolysed with 0.25M-sulphuric acid (100 ml) at 100° for 9h [CM 3b]. The neutralisate (BaCO₃) was concentrated to a small volume (15 ml). This contained 680 mg carbohydrate, and oligouronic acids therein were precipitated in ethanol (5 - 6 vol) and removed by filtration. The filtrate was again concentrated to a small volume (5 ml) and additional oligosaccharides were precipitated as before (total barium uronates 968 mg). The final filtrate was concentrated to dryness. The residue (112 mg carbohydrate) was dissolved in water and the solution was treated with IR 120(H⁺) and IR 45(OH⁻) resins.

To an aliquot (ca. 30 mg carbohydrate) of the resulting solution, glucose oxidase (Glucostat XI) was added to convert the glucose into gluconic acid. After 1.0 h the enzyme was removed by coagulation in methanol at 40° and filtration. The residual galactose and mannose in the filtrate were separated on 3MM paper [CM 5(a)i]. After elution of
the individual bands, the amount of each sugar was measured by the phenol-sulphuric acid method \([GM\ 2a]\). Each sugar was tested for purity by paper chromatography in several solvents and the identity of the galactose confirmed as the D-sugar with galactose oxidase (solvent \([GM\ 5(a)\ i]\) and spray \([GM\ 5(b)\ vi]\)). The identity of each of the two sugars was further confirmed on GLC as the hexose-TMS and hexitol-TMS derivatives \([GM\ 4(e)\ and\ 5(f)\ ^{y}]\).

The rest of the neutral solution was treated as before to recover more mannose and the phenylhydrazones of the separated mannose and of authentic D-mannose were prepared. The melting points of the two derivatives and a mixed melting point were determined.

(e) **Determination of the sugar proportions in different acid hydrolysates**

The polysaccharide (4 samples: 7.9 mg, 18.5 mg, 20.2 mg 31.0 mg) was hydrolysed with formic acid \([GM\ 3a]\) for 4, 6, 8, and 12 h respectively. A third of the hydrolysate was converted into the hexaalditol acetate \([GM\ 4c,\ f]\) and examined by GLC \([GM\ 5\ (f)\ (ii)\ a]\). The peak areas were determined by an electronic integrator coupled to the chromatograph.

The rest of the hydrolysates were each taken to dryness, esterified, reduced and hydrolysed \([GM\ 4g]\) for 4 h, the first three samples with formic acid \([GM\ 3a]\) and the fourth sample with 0.5M-sulphuric acid \([GM\ 3b]\). After examination on paper (solvent \([GM\ 5(a)\ i]\) and spray \([GM\ 5(b)\ i]\), each was converted into the hexaalditol acetate and examined on GLC as above.
(f) Characterisation of the uronic acid

An aliquot of the formic acid hydrolysate of the polysaccharide was examined by paper chromatography in solvents [GM 5(a) iii, ix]. A further aliquot of the hydrolysate was esterified, reduced, and hydrolysed [GM 4g] and examined on paper (solvents [GM 5(a) i-iv] and sprays [GM 5(b) i,ii]). A third portion of the hydrolysate was separated into neutral and acidic material [GM 4k]. The acid fraction was further separated on preparative paper [GM 5(a) i]. Both the lactone and the acid bands were examined by chromatography (solvents [GM 5(a) i, iii, ix]) and by ionophoresis in borate buffer containing calcium ions [GM 5(d)iii]. Each was then esterified, reduced, hydrolysed [GM 4g] and examined on paper (solvents GM 5(a) i, ii, iv] and sprays [GM 5(b) i, ii, v]). The neutral sugar obtained after reduction was also examined by GLC as the sugar-TMS, alditol-TMS, and alditol acetate [GM 5f].

Expt. 4. Determination of glucuronic acid content

The polysaccharide (7.1 mg in water, 20 ml) and the HTAB purified polysaccharide [expt. 5(f) ii] (6.2 mg in water, 20 ml) were each homogenised by shaking for 36 h. Aliquots (0.5 ml and 1 ml) in duplicate were used for the uronic acid determination by the carbazole method [GM 2(b)ii].

The polysaccharide (11.5 mg in water, 10 ml) and a formic acid hydrolysate of the polysaccharide (11.3 mg in water, 10 ml) were assayed for uronic acid content by the meta-hydroxydiphenyl method [GM 2(b) iii]. Four aliquots (0.2 ml each) were used from both materials.
Expt. 5. Attempted fractionation of the polysaccharide

(a) Barium hydroxide precipitation:— To a solution of the polysaccharide (50 mg in water, 200 ml), saturated barium hydroxide solution (10 ml) was added with constant stirring. Precipitation was allowed to proceed overnight, and after filtration, the filtrate was examined for carbohydrate content and then analysed by paper chromatography after acid hydrolysis.

(b) With dimethylsulphoxide (DMSO):— The polysaccharide (51 mg) was shaken with dry DMSO (4 ml) for 1 week. The dispersion was allowed to stand for 1 week whence a supernatant and precipitate separated. The mixture was centrifuged to give a DMSO-soluble and a DMSO-insoluble fraction.

The DMSO-soluble fraction was diluted with water, extensively dialysed, examined for carbohydrate content [GM 2a], freeze-dried and weighed. An aliquot (5.5 mg) was hydrolysed [GM 3a] and examined on paper (solvent [GM 5(a) iv] and sprays [GM 5(b) i, ii]), and by GLC [GM 5(f)(i) v]. Another aliquot (20 mg) was methylated [GM 4h] and analysed as methylated alditol acetates and methylglycosides by GLC and GC-MS.

The DMSO-insoluble residue was dispersed in water (40 ml) and dialysed. It was centrifuged to give two fractions, one which was soluble in water and one which was not. Each was examined for carbohydrate content, freeze-dried, weighed, hydrolysed with formic acid [GM 3a] and examined on paper and by GLC as above.
(c) **Analytical ultracentrifugation:**— (i) An aqueous solution of the polysaccharide (0.3% w/v) was spun at 40,000 rpm and 60,000 rpm on a Spinco analytical ultracentrifuge Model E. One millilitre capacity cell with Schlieren pattern was used. Running times were 3h each.

(ii) The polysaccharide was partially methylated by the Hakomori method [GM 4h]. The methylated product was dissolved in dry chloroform, and the solution (1.0% w/v) was spun at 60,000 rpm for 3h as above.

(d) **With hexadecyltrimethylammonium bromide (HTAB):**— 3% HTAB solution (Eastman Kodak) (50 ml) was slowly added to a solution of the polysaccharide (500 mg in water, 260 ml) with vigorous stirring for 24 h.

The mixture was centrifuged at 6,000 rpm for 3 h. The supernatant was treated with potassium iodide to precipitate excess HTAB. This was removed by filtration on glass fibre paper and the filtrate was further treated with potassium iodide (in all 3 g). After filtration, the filtrate was treated with Amberlite resins IR 120 (H⁺) and IR 400 (Ac⁻). After concentration, it was assayed for carbohydrate content [GM 2a] and examined by paper chromatography.

The complex was decomposed in 1% sodium chloride (400 ml, 60 h with stirring). The polysaccharide was recovered by precipitation in 6 volumes of acetone (this was chosen after trial runs with alcohol/acetone mixtures). The procedure was repeated 3 times to remove adhering HTAB and the polysaccharide finally recovered by dialysis and freeze-drying (425 mg). It was assayed for carbohydrate [GM 2a],
hydrolysed with formic acid, and examined on paper and as TMS-derivatives, by GLC. It was also assayed for uronic acid

[GM 2(b)ii].

**Expt. 6. Proton magnetic resonance (NMR) of the polysaccharide**

The polysaccharide in the free acid form obtained after treatment with Amberlite IR 120 (H⁺) resin and extensive dialysis was freeze-dried. The freeze-dried material (30 mg) was subjected to deuterium exchange by shaking in D₂O for 24 h and freeze-drying. This was repeated twice. The thick gel was spun in a Varian anaspect model EM 360 spectrometer for 5 min before taking its spectrum. Internal standard (trimethylsilane) was used for reference.

The residual polymer after autohydrolysis (see expt 7) was also subjected to deuterium exchange and its NMR spectrum was taken similarly.

**Expt. 7. Autohydrolysis of the free-acid polysaccharide**

The polysaccharide (1.26 g) was shaken with water (300 ml) for 3 days to form a homogeneous gel. To this was added Amberlite IR 120 (H⁺) resin (300 ml) and the mixture was shaken for 12 h. After centrifugation and decantation, the decanted gel and washings (90 ml altogether) was autohydrolysed in a sealed atmosphere of nitrogen at 100°C for 10 h.

**Expt. 8. Ethereal extraction of the autohydrolysate**

The autohydrolysate was extracted with ether (100 ml) for 5 h. The extraction was repeated twice more with fresh ether. The combined ethereal extracts was concentrated to about 3 ml.
Expt. 9. Identification and characterisation of acetic acid

(a) Identification

The ethereal extract was analysed by paper chromatography in neutral solvent \([\text{GM 5(a) iv}]\) on Whatman No. 1, 3M and No. 17 paper (spray \([\text{GM 5(b) ix}]\)). Freshly distilled pyruvic acid was used as standard.

Thin layer chromatography was carried out on polygram Sil G plate in two solvents \([\text{GM 5(e) i, iii}]\). Standard and development were as above.

Paper electrophoresis was performed as Whatman No. 3 paper in pyridine/acetic acid buffer (pH 6.7) \([\text{GM 5(d) iv}]\) for 30 min at 1000 volts/20 mA. Standard and development were as above.

Infra red spectroscopy \([\text{GM 5 h}]\) was carried out on a mixture of ground KCl and ethereal extract carefully dried at 37° under reduced pressure.

The 2,4-dinitrophenylhydrazone derivative was prepared by the method of R.L. Shriner et al. Standard pyruvic acid-DNP was easily made, however several attempts on the ethereal extract failed to give any crystals. The derivatives were therefore examined by TLC on polygram Sil G in ethyl acetate-petroleum ether (60-80°) - acetic acid (50:50:7).

(b) Characterisation

The polysaccharide (50 mg, dry) was boiled at 100° for 30 min together with anhydrous N-methanolic hydrogen chloride (1 ml) in a sealed tube. This was opened after cooling and just before injection into the GLC carbowax 1500 column \([\text{GM 5(f)(ii)b}]\). Sodium acetate and sodium pyruvate were similarly treated.
The polysaccharide (free-acid form, 0.3 g in water, 100 ml) was autohydrolysed for 10 h. After cooling to room temperature, ether (25 ml) was added with stirring and the polysaccharide was precipitated in ethanol (250 ml). After filtration, the filtrate was neutralised to pH 7 with 0.1N-NaOH and evaporated to dryness.

The derived white solid was dissolved in D$_2$O and its NMR spectrum taken. Similarly the NMR spectrum of sodium acetate was taken.

A drop of the solution was also converted into the hydroxamate derivative by adding 3 drops of saturated alcoholic hydroxylamine hydrochloride, 3 drops of 20% methanolic potash, boiling and acidifying with 0.5N-HCl after cooling, the derivative was examined by TLC in (i) n-pentanol-formic acid-water (organic layer) (ii) water-saturated phenol. Development of the chromatograms was by spraying with ferric chloride (FeCl$_3$, 1g; conc HCl, 0.1 ml; 95% ethanol, 100 ml).

The polysaccharide (2 g in water 2000 ml) was reduced with KBH$_4$ (0.5 g) overnight. N-sodium hydroxide (500 ml) and KBH$_4$ (1.5 g) were added while the solution was stirred under nitrogen. After 24 h, the mixture was extensively dialysed to give a deacetylated polysaccharide.

The dialysate (15 L) from above was concentrated to 500 ml, brought to pH 4.5 with 5N-sulphuric acid and steam distilled. The steam distillate was carefully neutralised with 0.1N-NaOH, and concentrated to a small volume.

The p-nitrobenzyl derivative was prepared by the method of E.E. Reid et al. Sodium acetate was similarly treated. The p-nitrobenzyl derivative of the unknown had to be extracted into ethanol,
treated with IR 120 (H⁺), evaporated to dryness and dissolved in alcohol for crystallisation. Melting point and mixed melting point of the derivatives were determined.

Expt. 10. Estimation of acetyl content

(a) The autohydrolysate (see expt. 7) was dialysed in a closed system against a water/alcohol solution (1000 ml/600 ml). The dialysis sac content was freeze-dried and weighed. From the weight of the original polysaccharide and the weight of the deacetylated polymer, the percentage weight of acetic acid was calculated.

The dialysate was concentrated to 330 ml, and aliquots (25 ml) were titrated against 0.1N-NaOH in an atmosphere of nitrogen. Phenolphthalein was used as indicator.

(b) The acetate contents of the purified polysaccharide (19.7 mg in buffer, 20 ml) and (35.5 mg in buffer, 25 ml) and the HTAB-purified polysaccharide (11 mg in buffer, 10 ml) as well as the deacetylated polymer (8.4 mg in buffer, 5 ml) were determined. Several aliquots (1 ml) of each solution were used in the determinations according to [GM 2f].

Expt. 11. Estimation of pyruvic acid content

Determinations [GM 2h] were carried out on (a) the ethereal extract (see expt. 8) after dissolution in water, (b) the dialysate (see expt. 10), (c) the sodium salt solution (see expt 9b). To check for inhibition by the polysaccharide, standard pyruvic acid solutions containing the original polysaccharide or the residual polymer after autohydrolysis were assayed.
Expt. 12. Location of O-acetyl residues

The dried polysaccharide (60 mg) was homogenised in dry dimethyl sulfoxide (2 ml) by shaking for 48 h. The mixture was kept at -15°C, and dry p-toluenesulphonic acid (20 mg) was added, followed by methylvinylether (3 ml, condensed at ice/methanol temperature) with constant stirring.

After 3 h at -15°C, the clear red reaction mixture was eluted from a Sephadex LH 20 column (25 x 3 cm) with anhydrous acetone, under slight suction. This failed to achieve any separation, and the experiment was repeated. After 3 h at -15°C and 14 days at 4°C, the reaction mixture was centrifuged. The centrifugate (B1 fraction) was washed with acetone, and after methylation, the methylated product gave a chloroform-soluble fraction (B1-1) and insoluble fraction (B1-2).

The supernatant and acetone washings were concentrated (10 ml) and kept at 4°C for 2 days. The mixture was again centrifuged to give the centrifugate (B2). The supernatant (B3) was poured into water (200 ml), dialysed and the mixture from the dialysis sac was concentrated to dryness. After methylation [GM 4h], the fractions were each analysed as methylated alditol acetates on GLC and GC-MS [GM5 f, g].

Expt. 13. Linkage analysis of the neutral sugar components

The polysaccharide (24.5 mg) was methylated by the Hakomori method [GM 4h]. The methylated product was analysed for carbohydrate content [GM 2a], hydrolysed with formic acid [GM 3a] and the hydrolysate assayed for carbohydrate content. Half of the hydrolysate was converted into the methylglycosides [GM 4d] and analysed by GLC [GM 5(f) i] α,β. The other half was reduced with borohydride
[GM 4c] and converted into alditol acetates [GM 4f] for analysis by GLC and GC-MS [GM 5(f) (ii) a and 5(g)i] and the areas of the different peaks were measured with an electronic integrator.

The polysaccharide (30.6 mg) was methylated as above, and hydrolysed with 0.5N-H₂SO₄ [GM 3(b)i]. The hydrolysate was examined as methylglycosides and alditol acetates as before.

Expt. 14. Attempts to determine the uronic acid linkage(s).

(a) The polysaccharide (4 aliquots A, B, C, D) (ca. 30 mg) was methylated twice as in [GM 4h]. Unless otherwise stated below the final product was hydrolysed with formic acid and examined as above.

Sample A was reduced with lithium aluminium hydride [GM 4n] and re-methylated [GM 4h].

Sample B was subjected to a second Hakomori methylation and then reduced with borohydride in methanol/water [GM 4c].

Sample C was methanolysed [GM 4b], and reduced with LiAlH₄ [GM 4n].

Sample D was hydrolysed with formic acid [GM 3a]. Half of the hydrolysate was examined as above. The rest was esterified [GM 4d] reduced with borohydride [GM 4c], hydrolysed [GM 3a] and treated as above.

(b) The uronic acid linkage was determined by methylating [GM 4h] (i) the residual polymer (12.7 mg) obtained after partial acid hydrolysis (see expt. 20b), (ii) the neutral polymer (20 mg) obtained after carboxyl-reduction of the carbodiimide complex of this residual polymer (see expt. 20b). The methylated products were each hydrolysed with formic acid and examined as in expt. 13.
Expt. 15. Determination of ultrasonic effect and carbanion effect during methylation

The polysaccharide (3 aliquots, ca. 30 mg) was methylated (a) by the usual Hakomori method [GM 4h], (b) as per [GM 4h], but for the replacement of the ultrasonic bath by a mechanical shaker, (c) as per [GM 4h] and the methylated product was re-methylated [GM 4h]. In all three cases, the final products were examined as in expt. 13.

Expt. 16. Periodate oxidation of the polysaccharide

The polysaccharide (0.5 g in triplicate A, B, C) was oxidised with periodate [GM 4t] for 4, 4 and 2 days respectively and the extent of oxidation monitored by spectroscopic method and by titration. The derived polyaldehyde was reduced in borate buffer (pH 9) [GM 4(m)ii]. The polyalcohols (A, B, C) were each freeze-dried, weighed and assayed for carbohydrate content [GM 2a]. They were each separately hydrolysed with formic acid, examined on paper (solvents [GM 5(a)i,ii] and spray [GM 5(b)i]) and as sugar-TMS derivatives by GLC [GM 5f]. Polyalcohol C was further examined for nitrogen content [GM 2i] and for uronic acid content [GM 2(b)ii].

Expt. 17. Sequential oxidations of the polysaccharide

The polysaccharide (1.304 g) was oxidised with periodate for 2 days [GM 4t]. The derived polyaldehyde was reduced with sodium borohydride [GM 4(m)i]. The derived polyalcohol (D1) was freeze-dried and weighed. It was examined, after acid hydrolysis [GM 3a], on paper (solvents [GM 5(a) i-iii] and sprays [GM 5(b) i-ii]), and by GLC [GM 5f] of the sugar-TMS and alditol-TMS derivatives. The polyalcohol (D1) was methylated and examined as methylated methylglycosides and alditol acetals by GLC and GC-MS [GM 4h, 5f, 5g].
Polyalcohol (D1) (500 mg) was subjected to periodate oxidation [GM 44] [GM 4(m)i] and the recovered polyalcohol (D2) (275 mg) was examined as above by acid hydrolysis, methylation and chromatography. The polyalcohol (D2) (250 mg) was re-oxidised as above and the final polyalcohol (D3) (30 mg) was examined, after acid hydrolysis and chromatography and the extent of oxidation monitored by spectroscopic method and by titration (see table 9, p93). The hydrolysate was further examined by paper electrophoresis [GM 5(d) iv], using glucuronic acid, glyceric acid and erythronic acid as standards.

Expt. 18. Oxidations of the deacetylated polysaccharide

The deacetylated material (see exp. 9), (0.5 g in duplicate) was oxidised with periodate for 6 days. [GM 44, 4(m)ii]. The derived polyalcohol (E1) was examined for carbohydrate content [GM 2a], uronic acid content [GM 2(b) ii] and after acid hydrolysis [GM 3a] on paper [GM 5(a) ii, 5(b) i], and by GLC as sugar-TMS derivatives [GM5f(i)].

The polyalcohol (E1) (278.1 mg) was oxidised with periodate for 51 hours as above. The recovered polyalcohol (E2) was examined for carbohydrate content, and by acid hydrolysis and paper chromatography [GM 3a, 5(a) ii, 5(b)i].

Expt. 19. Oxidations of the carboxyl-reduced polysaccharide

The carboxyl-reduced polysaccharide (see exp. 20b) (74.8 mg in water, 25 ml) was oxidised with periodate (0.003M, 25 ml) for 55 h [GM 44, 4(m) i]. An aliquot of the recovered polyalcohol (G1) (5 mg) was hydrolysed with formic acid [GM 3a], examined on paper [GM 5(a) i, 5(b), i, ii] and by GLC [GM 5(f)(ii)a] as hexalditol acetate [GM 4c,f].
The rest of the polyalcohol (G1) (23.2 mg) was periodate-oxidised as above; and an aliquot of the derived polyalcohol (G2) (5 mg) was hydrolysed and examined as above. Additional spray used was glucose oxidase [GM 5(b)v]. The polyalcohol (G2) (10.1 mg) was also methylated and examined as methylated methylglycosides and alditol acetates by GLC and GC-MS [GM 4h; 5f, g].

Expt. 20. Partial acid hydrolysis

(a) **Trial runs**:- The polysaccharide (200 mg) was hydrolysed with 0.25M-oxalic acid (200 ml) at 100°. Aliquots (50 ml) were removed after 1, 2, 4 and 5 h, and each was poured into ethanol (6 vol). After centrifugation of the mixture, the centrifugate (dried) was weighed. The supernatant was neutralised [GM 3c] and examined on paper (solvents [GM 5(a)ii, iv] and spray [GM 5(b)i]).

(b) **Large scale partial hydrolysis**:- The crude polysaccharide (4.8 g carbohydrate) was hydrolysed with 0.25M-oxalic acid for 5h at 100°. The mixture was precipitated in ethanol and worked up as above. The derived alcohol soluble material after neutralisation was examined on paper (solvent [GM 5(a) iv] and sprays [GM 5(b) i, ii, v]) and by electrophoresis [GM 5(d) iv].

The precipitate was redissolved in water and dialysed. The residual polymer (retentate) was examined for carbohydrate content [GM 2a], for uronic acid content [GM 2(b)ii], and after acid hydrolysis [GM 3a] by paper chromatography as above. Assay for carbohydrate content was also carried out on the dialysate.

The retentate (233 mg) was complexed with ethyl-3-dimethylamino-propyl carbodiimide hydrochloride (EDC) and reduced with borohydride
by the method of R.L. Taylor and H.E. Conrad to give a neutral polymer. This was examined in expt. 14b(ii) and expt. 19. An aliquot (10 mg) was also examined after formic acid hydrolysis [CM 3a] by paper chromatography as above and by GLC of the hexaacetates [CM 5(f) (ii) a] and the peak areas measured.

(c) **Separation into acidic and neutral fractions on Deacidite column**

An aliquot of the derived neutralisate (2.2 g carbohydrate) was converted into the free-acid form by shaking with Amberlite IR 120 (H⁺) resin and the recovered material (1.17 g carbohydrate) was eluted from a Deacidite column (Bed volume, 400 ml) [CM 4k] to yield a neutral fraction (460 mg carbohydrate) and an acidic fraction (710 mg carbohydrate).

(d) **Fractionation of the neutral and acidic fractions**

The neutral and acidic (as ammonium salts) fractions were further fractionated by preparative paper chromatography [CM 5c] in solvents [CM 5(a) iv] and [CM 5(a) i] respectively. Each sugar band eluted was assayed for carbohydrate content and further separated on paper to obtain a pure product.

**Expt. 21. Examination of the neutral oligosaccharides**

(a) The neutral fraction F1 \( R_{glc} = 0.56 \) in solvent [CM 5(a) iv] was examined for homogeneity on paper [CM 5(a) iv] solvent and [CM 5(b) i, ii, vii, viii], for specific optical rotation \( (c = 0.385 \text{ g/100 ml}) [CM 2g] \), and by paper chromatography after acid hydrolysis, [CM 3(b) iii] (solvent [CM 5(a) ii, iv] and spray [CM 5(b) i, ii, v, vi]). The hydrolysate was also examined by electrophoresis in molybdate buffer (pH 5) [CM 5(d)i].
F1 (8 mg) was methylated \( [\text{GM 4i}] \) and examined as partially methylated alditol acetates by GLC and GC-MS \( [\text{GM 4c, f; 5f, g}] \). F1 (4 mg) was reduced with borohydride \( [\text{GM 4c}] \) and examined by electrophoresis \( [\text{GM 5(d) i}] \) using maltitol as reference.

F1 (8 mg) was reduced with sodium borodeuteride \( (D^4) \) \( [\text{GM 4c}] \) in \( D_2O \). The derived disaccharide alditols were methylated \( [\text{GM 4i}] \) and examined by GLC and GC-MS \( [\text{GM 5(f) ii, iY; GM 5(g) i, ii}] \) as permethylated disaccharide alditols.

(b) The neutral fraction F2 \( (R_{glc} = 0.44 \text{ in solvent } [\text{GM 5(a) iv}]) \) was examined for homogeneity by chromatography \( [\text{GM 5(a) iv}] \), for carbohydrate content, for DP \( [\text{GM 2g}] \), and after acid hydrolysis (1 mg) \( [\text{GM 3(b) iii}] \) by chromatography \( [\text{GM 5(a) ii} \text{ and [GM 5(b) i, ii, v, vi}].

F2 (1.1 mg) was reduced with borohydride \( [\text{GM 4c}] \) and analysed by electrophoresis in molybdate buffer \( [\text{GM 5(d) i}] \).

(c) The neutral fraction F3 \( (R_{glc} = 0.36 \text{ in solvent } [\text{GM 5(a) iv}]) \) was reduced with sodium borohydride \( [\text{GM 4c}] \) and analysed by electrophoresis in molybdate buffer \( [\text{GM 5(d) i}] \).

(d) The neutral fraction F4 \( (R_{glc} = 0.25 \text{ in solvent } [\text{GM 5(a) iv}]) \) was examined for homogeneity, carbohydrate content, and DP as in (b) above. The optical rotation was determined. F4 (1 mg) was partially hydrolysed with formic acid (1 h) \( [\text{GM 3a}] \). The partial hydrolysate was examined on paper \( [\text{GM 5(a) iv} \text{ and [GM 5(b) i, vii, viii}].

F4 (2 mg) was reduced with borohydride \( [\text{GM4c}] \), and examined by electrophoresis \( [\text{GM 5(d) i}] \); and after acid hydrolysis \( [\text{GM 3a}] \) (2 h) the hydrolysate was examined on paper \( (\text{solvent [GM 5(a) ii, vii]} \text{ and sprays [GM 5(b) i, ii}]) \).
F4 (1.8 mg) was reduced with borohydride [GM 4c], and separated into F4a and F4b by electrophoresis [GM 5(d) i]. The derived complexes were each decomposed with 4N-NaOH (2 drops), the solution shaken with Biodeminrolit and evaporated to dryness. F4a was re-examined by electrophoresis [GM 5(d) i] before hydrolysis with formic acid (2 h) [GM 3a]. The hydrolysate was examined on paper [GM 5(a), ii, vii] and [GM 5(b) iii].

(e) The neutral fractions F5 and F6 (R$$^{\text{glc}} = 0.14$$ and 0.06 respectively in solvent [GM 5(a) iv]) were each assayed for carbohydrate content, and for DP [GM 2g]. Each was partially hydrolysed with formic acid (1$$^{\frac{1}{2}}$$ h) [GM 3a], and examined on paper [GM 5(a) iv, 5(b)i].

Expt. 22. Examination of the acidic oligosaccharides

(a) The acidic fraction G1 ($$R_{\text{glc}}$$ UA = 1 in solvent [GM 5(a) i]) was examined by electrophoresis in pyridine/acetic acid buffer (pH 6.7) [GM 5(d) iv], assayed for DP [GM 2g], and examined on paper (solvents [GM 5(a) i, ii, iv] and sprays [GM 5(b) i, ii, v]) both before and after acid hydrolysis (2 mg) [GM 3a].

G1 (2.1 mg) was reduced with borohydride [GM 4c], esterified [GM 4d], and reduced with borohydride [GM 4c]. The derived product was examined, both before and after acid hydrolysis [GM 3a], by electrophoresis [GM 5(d) iv] and by paper chromatography (solvents [GM 5(a) ii, vii] and sprays [GM 5(b) i, ii]).

(b) The acidic fraction G2 ($$R_{\text{glc}}$$ UA = 0.4 in solvent [GM 5(a) i]) was tested for homogeneity on paper (solvent [GM 5(a) ii,iv]), assayed for DP [GM 2g] and examined by electrophoresis [GM 5(d) iv].
G2 (2 mg) was hydrolysed with formic acid (4 h) \[\text{GM 3a}\] and examined on paper \[\text{GM 5(a) i, ii, iv}\] and \[\text{GM 5(b) i, ii}\].

G2' (5 mg) was esterified with methanolic hydrogen chloride (2\%) \[\text{GM 4b}\], reduced with borohydride \[\text{GM 4c}\] and hydrolysed \[\text{GM 3a}\]. The hydrolysate was examined on paper \[\text{GM 5(a) i, ii}\] and \[\text{GM 5(b) i, ii}\].

G2 (4 mg) was reduced with borodeuteride (D\(_4\)) \[\text{GM 4c}\], methylated by the modified Kuhn method \[\text{GM 4i}\], reduced with borohydride and after re-methylation, examined as permethylated alditol by GLC and GC-MC \[\text{GM 5(f) (ii)}\] and \[\text{GM 5(g) ii}\].

G2 (3 mg) was converted into the neutral disaccharide \[\text{GM 4a, c}\] and methylated by the modified Kuhn method \[\text{GM 4i}\] and hydrolysed with formic acid \[\text{GM 3a}\]. The hydrolysate was examined as methylated alditol acetates \[\text{GM 4c, f}\] \[\text{GM 5(f) (ii) a and 5(g)i}\].

(c) The acidic fraction G3 (R\(_{\text{glc U}}\) = 0.0) was examined by electrophoresis in pyridine/acetic acid buffer (pH 6.7) \[\text{GM 5(d) iv}\] and assayed for DP \[\text{GM 2g}\]. G3 (15 mg) was partially hydrolysed with formic acid (2 h) \[\text{GM 3a}\] and the partial hydrolysate was examined by paper chromatography (solvents \[\text{GM 5(a) i, ii, iv}\] and sprays \[\text{GM 5(b) i, ii, v}\]), and by electrophoresis \[\text{GM 5(d) iv}\].
RESULTS AND DISCUSSION

The crude polysaccharide: source, isolation and examination

The material was generously supplied by Alginate Industries Ltd., Girvan, Scotland. The bacterium, S19, used was tentatively identified as a plant pathogenic Xanthomonas species at the National Collection of Plant Pathogenic Bacteria, Ministry of Agriculture, Fisheries and Food, Plant Pathology Laboratory, Harpenden, England. S19 differs from most Xanthomonas species in that its pigment is orange rather than yellow.

The culture solution contained sucrose, acid hydrolysed casein, magnesium sulphate, d'potassium hydrogen phosphate and manganese sulphate in distilled water. The crude polysaccharide was obtained by precipitation from the culture solution in excess acetone. This was followed by filtration, air-drying and milling; and was supplied as a fawn powder.

The crude polysaccharide (dry matter 85%; viscosity of 1% dry matter solution 5,940 c.p.s.); after hydrolysis, gave glucose, uronic acid, galactose, and mannose together with trace amounts of fructose and rhamnose (paper chromatograms) (expt. 1).

Purification of the crude polysaccharide

The crude polysaccharide as a thick gel in water (ca. 3% by wt) was purified by alcohol precipitation and by extensive dialysis of the aqueous solution (expt. 2). The freeze-dried product was a white hydrophilic fluffy solid [73% recovery; carbohydrate content (as glucose) 70%; ash 6%; Protein 9.6%; [a]_D +233° (c, 0.006]. The presence of uronic acid was indicated by a positive naphthoresorcinol reaction.
Examination of the purified polysaccharide

The purified polysaccharide still dissolved with difficulty in water, and gave highly viscous solutions (expt. 3).

Hydrolysis with different concentrations of acid caused insignificant loss of carbohydrate (table 1).

Table 1

<table>
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<tr>
<th>Wt. of polysaccharide (mg)</th>
<th>Hydrolysis with</th>
<th>Carbohydrate content of polymer (mg)</th>
<th>Carbohydrate content of hydrolysate (mg)</th>
<th>% recovery</th>
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</tr>
<tr>
<td>147.0</td>
<td>90% HCOOH (6h)</td>
<td>102.9</td>
<td>102.0</td>
<td>100%</td>
</tr>
<tr>
<td>1128.9</td>
<td>0.25M-H₂SO₄(9h)</td>
<td>789.6</td>
<td>680.0</td>
<td>86%</td>
</tr>
</tbody>
</table>

Each of the hydrolysates gave spots on paper chromatograms corresponding to glucose, galactose, mannose and uronic acid together with varying proportions of oligosaccharides. The carboxyl reduced hydrolysate (expt. 3e) appeared to have a higher proportion of glucose. Electrophoresis in borate buffer (pH 10) confirmed these findings. Spots with M₆₁c 1.0, 0.9 and 0.7, the reported mobilities of glucose, galactose and mannose, were present, but the trace quantities of fructose and rhamnose present in the original material had been removed in the purification. Further confirmation of the presence of these three sugars was obtained from the gas-liquid chromatograms of the sugar-TMS, alditol-TMS and alditol acetates of the hydrolysates. The formic acid hydrolysate had [α]D +25° indicating a high proportion of...
D-isomers (cf. D-glucose +52°; D-mannose +14°; D-galactose +83°).

**Characterisation of the constituent sugars and uronic acid**

Glucose: This was not isolated from a hydrolysate but paper chromatography of the latter in four different solvents gave spots with the same mobility as authentic glucose run as control. After conversion of separate aliquots into the respective derivatives gas liquid chromatography gave peaks with retention times identical to those of glucose TMS, glucitol TMS, and glucitol hexacetate. Glucose oxidase spray, after paper chromatography confirmed the presence of D-glucose.

Galactose was identified, as for glucose, by paper and gas liquid chromatography. Because of the relatively large proportion of glucose in the hydrolysate it was necessary to remove this sugar before pure samples of galactose and mannose could be separated on 3MM paper. Accordingly the glucose in a hydrolysate was oxidised to gluconic acid and the remaining galactose and mannose were then separated. The former was characterised as D-galactose with galactose oxidase and by its $[\alpha]_D +74°$ (c, 0.08) (cf. lit. value +83°).

Mannose was identified as for the two previous sugars, and its identity confirmed by the preparation of crystalline mannose phenylhydrazone, m.p. and mixed m.p. with authentic D-mannose phenylhydrazone 199-200°.

Glucuronic acid was indicated by its paper chromatographic mobility, especially in solvent [GM 5(a) ix] which separates glucuronic acid from galacturonic acid, and by the presence of its lactone. The isolated lactone and uronic acid each gave a single spot, with the mobility of
glucuronic acid, on electrophoresis in borate buffer containing calcium ions. This technique separates glucuronic, galacturonic, mannnuronic and guluronic acids. The isolated acid after reduction gave only glucose when examined by paper chromatography and GLC as the TMS derivative. It was confirmed as the D-sugar with glucose oxidase proving the presence of D-glucuronic acid.

**Attempted fractionation of the polysaccharide**

At this stage it was essential to establish the homogeneity of the material. The high viscosity of a dilute aqueous solution made fractionation as a DE52 cellulose column and on a resin column impossible and other methods of fractionation were therefore investigated.

Preferential dissolution in dimethylsulphoxide was carried out and although the fractions set out in table 2 were separated, each was shown to contain the same sugars in approximately the same proportion (expt. 5).

### Table 2

<table>
<thead>
<tr>
<th>Fractions</th>
<th>Weight (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial S19 polysaccharide</td>
<td>51.0</td>
</tr>
<tr>
<td>DMSO-soluble polysaccharide</td>
<td>25.2</td>
</tr>
<tr>
<td>DMSO-insoluble, water-soluble polysaccharide</td>
<td>20.3</td>
</tr>
<tr>
<td>DMSO-insoluble, water-insoluble polysaccharide</td>
<td>3.7</td>
</tr>
<tr>
<td><strong>Total recovery</strong></td>
<td><strong>96%</strong></td>
</tr>
</tbody>
</table>

Methylation of the DMSO-soluble fraction gave similar results as methylation of the purified polysaccharide (page 62). It seems that a molecular weight fractionation is all that was achieved.
Fractional precipitation with barium hydroxide should leave any neutral polysaccharide in solution and complex with and precipitate acidic polysaccharide. With S19 it was found that about 3.4% of the starting material was left in solution. Analysis of this soluble material revealed the presence of glucuronic acid, indicating that it was low molecular weight material, rather than a neutral polysaccharide, which had been left in solution, and that S19 extracellular mucilage was devoid of a neutral polymer. This conclusion was substantiated by complex formation of S19 mucilage with hexadecyltrimethylammonium bromide (HTAB). This reagent is known to complex with acidic polysaccharides and to leave neutral polysaccharides in solution. About 85% by weight of the polysaccharide (carbohydrate content 71%) was recovered from the HTAB complex. Examination of the neutral solution remaining after removal of the HTAB complex and after removal of the excess HTAB revealed the presence of glycerol and trace quantities (0.06%) of oligosaccharides of the starting material and no polysaccharide.

The loss of about 15% by weight of the starting material could have occurred during the removal of HTAB from the supernatant and this could be neutral polysaccharide. The increase in the proportion of uronic acid 27%, as compared to 23%, (Table 3, p.75) in the polysaccharide recovered from the HTAB is in line with this conclusion. On the other hand, after cleavage of the complex repeated dissolution and precipitation is necessary to remove all the HTAB from the polysaccharide and this could account, at least in part, for the loss. Moreover the HTAB purified polysaccharide contained the same constituents in the same proportions as the alcohol purified polysaccharide.
Analytical centrifugation of a 1% aqueous solution of the polysaccharide failed to give any true boundary peak due to the gelling properties of the polymer. The same technique applied to a chloroform solution of the partially methylated polymer gave the following Schlieren patterns at 60,000 r.p.m.

![Schlieren patterns at 60,000 r.p.m.](image)

After 1 hour  
After 3 hours

The patterns show a homogeneous dispersion of fairly low molecular weight polymers possibly produced by degradation during methylation, and no indication of separate polysaccharides.

The above experiments all indicate S19 to be a single polydisperse acidic heteropolysaccharide free from any appreciable quantity of neutral polymer.

**Glucuronic acid content**

This was determined on the alcohol purified, the HTAB purified, and on a hydrolysate of the former material by the carbazole or the meta-hydroxydiphenyl methods (expt. 4). The weights were read off standard glucurone graphs and the results are given in table 3.
Table 3

<table>
<thead>
<tr>
<th>Sample of S19</th>
<th>Method</th>
<th>Uronic acid content</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alcohol purified</td>
<td>Carbazole(^a)</td>
<td>23%</td>
</tr>
<tr>
<td>HTAB purified</td>
<td>Carbazole(^a)</td>
<td>26.9%</td>
</tr>
<tr>
<td>Alcohol purified</td>
<td>(^m)-OH-(\beta)(^b)</td>
<td>24.6%</td>
</tr>
<tr>
<td>Hydrolysate of alcohol purified</td>
<td>(^m)-OH-(\beta)(^b)</td>
<td>26.0%</td>
</tr>
</tbody>
</table>

(a) \([\text{GM 2(b) ii]}\] (b) \([\text{GM 2(b) iii]}\]

Molar proportions of the component monosaccharides

The impossibility of completely hydrolysing the polysaccharide to the constituent monosaccharides without excessive degradation made accurate determination of their proportions extremely difficult. Hydrolysates with 90\% formic acid for 4, 6, 8 and 12 h (expt. 3e) were carried out and the molar proportions of the monosaccharides were determined by GLC of the peak areas of the hexaacetates (table 4) although each of the hydrolysates contained a fairly high proportion of oligouronic acids. In each of these hydrolysates glucose was present in greatest proportion and there was twice as much galactose as mannose.

The acid present in each of the hydrolysates was reduced to glucose and the resulting solution rehydrolysed for a further 4 h (table 4). Paper chromatography revealed the presence of oligosaccharides in the hydrolysates derived from the initial 4 h and 6 h hydrolysates.

It can be seen that the proportion of mannose has doubled in the reduced solutions indicating that it was probably held in glucuronosyl linkage in the polysaccharide and retained in oligouronic acids.
After reduction

Assuming that each of the constituents have been degraded to the same extent then the 8 h and 12 h reduced materials gave the best picture of the proportions of the constituents in the polysaccharide.

It must be remembered that the glucose has been derived not only from the glucose but also from the glucuronic acid present in the polysaccharide. This acid (as glucurone) comprises approximately 25% of the carbohydrate (see table 3) or about 1.6 molar parts of the glucose. It follows that the approximate ratio of GlcUA : Glc : Gal : Man is 1.6 : 3 : 1 : 1 in the polysaccharide.

The occurrence of acetate residues

Esters of acetate and pyruvate commonly occur in bacterial polysaccharides. Infrared spectra of the present polysaccharide gave a band at 1725 cm\(^{-1}\) indicative of such esters. (see IR spectrum p.77). The NMR spectrum of the initial polysaccharide supported this conclusion by showing a peak at 2.27 ppm. These esters are labile to autohydrolysis of the free acid form of the polysaccharide and indeed the NMR spectrum of the polysaccharide recovered after autohydrolysis was devoid of this peak.
Electrophoresis and infrared spectroscopy of the ethereal extract of the autohydrolysate (expt. 9) indicated the presence of a carboxylic acid (see IR spectrum p.79). The same extract gave a single spot $R_F$ 0.72 on a paper chromatogram (cf. pyruvic acid $R_F$ 0.72, acetic acid $R_F$ 0.72 run as controls), and $R_F$ 0.50 in ethylacetate and $R_F$ 0.83 in water/butanol, the latter two on thin layer. These techniques do not distinguish between acetic acid and pyruvic acid. The derived 2,4-dinitrophenylhydrazone of the ethereal extract, however, had $R_F$ 0.70 run on thin layer compared to $R_F$ 0.50 of the same derivative of pyruvic acid run as control.

GLC of the methyl ester derivative of the carboxylic acid gave a peak with the same retention time as that of methyl acetate. NMR-spectroscopy of the derived sodium salt gave a peak at 1.95 ppm identical with that of authentic sodium acetate. TLC of the hydroxamic acid derivative in two different solvents gave, in each case, a single spot of identical mobility to that given by the hydroxamic acid derivative of sodium acetate.

The presence of acetic acid was further confirmed by preparing the p-nitrobenzyl derivative which had m.p. and mixed m.p. with authentic p-nitrobenzyl acetate$^{201}$ 77-78°.

These results indicate the absence of pyruvic acid and this was confirmed with lactic acid dehydrogenase. Determination by the enzymic method (expt. 11) either on the ethereal extract, or on the dialysate of the autohydrolysate or on the sodium salt derivative showed the pyruvic acid content to be zero.
Acetate content

The acetate content of S19 polysaccharide, before and after purification with HTAB (expt. 10), was found to be 18.7\%, based on carbohydrate content or 1 mole of acetate for every 2 anhydro units. The deacetylated polymer was shown to contain no acetate by the same method of determination, showing also that the carboxyl group of the uronic acid residues in the polysaccharide did not affect the determination.

The acetate content was confirmed by comparing the weight of the polysaccharide (freeze-dried) before and after deacetylation:

\[
\begin{align*}
\text{Wt. of initial polysaccharide} & = 1.2739 \text{ g (W1)} \\
\text{Wt. of deacetylated polysaccharide} & = 1.037 \text{ g (W2)} \\
\text{\% of acetate} & = \frac{(W1 - W2)}{W1} = 18.5\%.
\end{align*}
\]

Titration of the dialysate after deacetylation, however, gave an acetate content of 8.8\% showing that some acetic acid has been lost due to its volatile nature.

Location of the O-acetyl residues.

The sites of O-acetyl residues in polysaccharides have been located by previous workers by first blocking the free hydroxyl groups with methyl vinyl ether. Simultaneous deacetylation and methylation of the product results in the acetyl groups being replaced with methyl groups. In the present experiment (expt. 12), hydrolysis gave a number of products including 2-O-methylglucose, 3-O-methylglucose, and 2,3-di-O-methyl-glucose. This indicates that the O-acetyl residues are present on the C-2 and/or C3 of the glucose residues. However, this evidence must be regarded as only tentative because the insolubility of the polysaccharide
made complete etherification with methyl vinyl ether impossible. Examination of the methylated products from the separated fractions B 1.1, B 1.2, B2 and B3 (see expt. 12) showed that B3, the acetalated polymer which was soluble in the reaction mixture, had most of its free hydroxyl groups blocked with methyl vinyl ether: since B3 was the only fraction that contained mono- and di-\(\beta\)-methylglucoses and no higher methylated sugars.

The experiment also showed that the acetalated polymer could be easily obtained free from reagents by dialysis against water instead of gel permeation chromatography on the more expensive Sephadex LH 20 column.

**Methylation Studies**

The polysaccharide was methylated by the Hakomori method and it can be seen (table 5) from the weight of methylated sugars present after hydrolysis that comparatively little carbohydrate has been lost during these procedures; particularly since methylated sugars respond less sensitively to the phenol-sulphuric acid test. Thus the hydrolysate is representative of the whole macromolecular structure and is not a fraction of the polysaccharide.

**Table 5**

<table>
<thead>
<tr>
<th>Carbohydrate as glucose (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>initial polysaccharide (24.5 mg)</td>
</tr>
<tr>
<td>methylated polysaccharide</td>
</tr>
<tr>
<td>methylated polysaccharide hydrolysate</td>
</tr>
</tbody>
</table>

(a) by the phenol-sulphuric acid method (487 nm).
Conversion of the methylated sugars into their methylglycosides, (expt. 13) gave the pattern of GLC peaks detailed in table 6. The identities of the methylated sugars, apart from the 3,4,6-tri-O-methyl-

<table>
<thead>
<tr>
<th>Position of O-methyl</th>
<th>methylglycosides of</th>
<th>Retention times of observed peaks</th>
</tr>
</thead>
<tbody>
<tr>
<td>2,3,4,6-</td>
<td>glucose</td>
<td>CM 5(f)(i)α 1.00; 1.38</td>
</tr>
<tr>
<td>3,4,6-</td>
<td>glucose</td>
<td>CM 5(f)(i)β 1.00; 1.35</td>
</tr>
<tr>
<td>2,4,6-</td>
<td>galactose</td>
<td>CM 5(f)(i)α 2.90; 4.30</td>
</tr>
<tr>
<td>2,3,6-</td>
<td>glucose</td>
<td>CM 5(f)(i)β 1.72; 2.2</td>
</tr>
<tr>
<td>3,6-</td>
<td>mannose</td>
<td>CM 5(f)(i)α 3.20; 4.30</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CM 5(f)(i)β 3.2; 3.8</td>
</tr>
</tbody>
</table>

sugar, were confirmed by the retention times of the respective standards. Authentic methyl 3,4,6-tri-O-methylglucoside gives peaks with retention times of 2.90 and 4.30 (10% EDS), and 1.72 (15% PPE), the same derivative of mannose has retention times of 2.90 (10% EDS) and 1.72 (15% PPE); whereas the peak with retention time 4.30 should serve to characterise this material as the 3,4,6-tri-O-methylglucose derivative, the presence in the hydrolysate of 2,4,6-tri-O-methylgalactose and 2,3,6-tri-O-methylglucose both of which have peaks with retention time of 4.30 makes it impossible to decide whether it is a derivative of glucose or mannose.

Conversion of the methylated sugars in the hydrolysate into the corresponding alditol acetates and examination by GLC gave the pattern of peaks detailed in table 7, and the mass spectra of each of these peaks are shown on the following pages.
Table 7

<table>
<thead>
<tr>
<th>Position of O-methyl</th>
<th>Alditol acetate derivative of</th>
<th>Retention times of observed peaks (OV 225 column)</th>
<th>Relative peak area ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>2,3,4,6-</td>
<td>glucose</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>3,4,6-</td>
<td>glucose</td>
<td>1.7</td>
<td>1.1</td>
</tr>
<tr>
<td>2,4,6-</td>
<td>galactose</td>
<td>1.9</td>
<td>1.0</td>
</tr>
<tr>
<td>2,3,6-</td>
<td>glucose</td>
<td>2.15</td>
<td>1.7</td>
</tr>
<tr>
<td>3,6-</td>
<td>mannose</td>
<td>3.2</td>
<td>1.0</td>
</tr>
</tbody>
</table>

These results are in agreement with those deduced from the methylated glycosides. Unfortunately, the 3,4,6-tri-O-methyl derivatives of glucitol and mannitol (as acetates) each gives a peak with retention time 1.7; and since the mass spectra do not distinguish between the different hexitols it is impossible from these facts to say which of these two sugars is present as the 3,4,6-tri-O-methyl derivative or whether it is a mixture of the two. This will be discussed later in the light of other evidence.

The relative peak area ratios of the different methylated sugars given in Table 7, p. 83 give the same proportion of end group as branch point but it must be remembered that these can only be regarded as very approximate quantitative proportions of the sugars since the more highly acetylated derivatives give relatively larger peaks. Furthermore the hydrolysate also contains a small proportion of oligouronic acids.
1,2,5-Tris-(2-acyl-3,4,6-tri-2-methyl octyl)}
1,3,5-tri-O-acetyl-2,4,6-tri-O-methyl hexitol

So far no evidence has been advanced for the linkage of the uronic acid residues. No methylated glucuronic acid derivatives could be identified in the hydrolysate. Since the polysaccharide had been subjected to two treatments with the dimethyl sulphinyl carbanion this is to be expected. Such units, particularly if 1,4-linked, suffer \( \beta \)-elimination reaction during the second treatment and on acid hydrolysis would be degraded (Fig. 1). The derived 4,5-unsaturated
Uronic acid residue is labile and hydrolyses to give 4-deoxy-5-ulosuronate which may react further to give a furan derivative.

It was hoped by reduction of the uronic acid to glucose with borohydride or lithium aluminium hydride either before or after partial methylation to provide evidence of the acid linkages [expt. 14(a)]. However in no case was complete reduction of the acid units achieved. Those units which had not been carboxyl-reduced underwent β-elimination. At the same time, cleavage of the uronosyl link appeared to occur since in each experiment, the proportion of 3,4,6-tri-O-methyl hexose increased with concomitant loss of 3,6-di-O-methylmannose. No definite conclusions regarding the glucuronic acid linkages could be drawn from these experiments.

In view of these results the uronic acid in a partly hydrolysed polysaccharide [expt. 14(b)] was converted into the carbodiimide complex before reduction with borohydride. In this case complete reduction was achieved. Comparison of the hydrolysates of the methylated partly hydrolysed polysaccharide and the methylated neutral polysaccharide derived from it showed, as the only difference, twice as much 2,3,6-tri-O-methylglucose in the latter. This proved that at least a high proportion of the uronic units are 1,4-linked.

A summary of the linkages in S19 polysaccharide is given in Table 8.
Table 8 Summary of linkages in S19 polysaccharide

<table>
<thead>
<tr>
<th>Sugar residues</th>
<th>Linkages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>end-group (non-reducing)</td>
</tr>
<tr>
<td></td>
<td>1,2- and</td>
</tr>
<tr>
<td></td>
<td>1,4-</td>
</tr>
<tr>
<td>Galactose</td>
<td>1,3-</td>
</tr>
<tr>
<td>Mannose</td>
<td>1,2,4 (branching)</td>
</tr>
<tr>
<td>Glucuronic acid</td>
<td>1,4-</td>
</tr>
</tbody>
</table>

To confirm the occurrence of $\beta$-elimination during methylation, the methylated product was re-methylated [expt. 15 (c)], and the results showed considerable reduction of the $3,6\text{-di-O-}$-methylmannose with the production of an increased proportion of $3,4,6\text{-tri-O-}$-methylhexose (see chromatograms, p. 92).

Furthermore, methylated polysaccharides obtained by the Hakomori method using either ultrasonification or shaking gave identical GLC chromatograms and mass-spectra. This shows that ultrasonification here does not cause excessive depolymerisation. 215
Gas-liquid chromatograms of permethylated alditol acetates:

Top: Before remethylation
Below: After remethylation

<table>
<thead>
<tr>
<th>Peaks</th>
<th>Position of O-methyl</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>2, 3, 4, 6</td>
</tr>
<tr>
<td>B</td>
<td>3, 4, 6</td>
</tr>
<tr>
<td>C</td>
<td>2, 4, 6</td>
</tr>
<tr>
<td>D</td>
<td>2, 3, 6</td>
</tr>
<tr>
<td>E</td>
<td>3, 6</td>
</tr>
</tbody>
</table>

Peaks E

Peaks C

Peaks D

Peaks B

Peaks A
Periodate oxidation studies

The results of periodate oxidations are tabulated below.

<table>
<thead>
<tr>
<th>Expt. No. and sample</th>
<th>Polyalcohol and yield</th>
<th>No. of days of oxidation content</th>
<th>Carbohydrate content</th>
<th>Monitoring method (see [GM 44])</th>
</tr>
</thead>
<tbody>
<tr>
<td>16 initial polysaccharide</td>
<td>A (60%)</td>
<td>4</td>
<td>83%</td>
<td>Thiosulphate</td>
</tr>
<tr>
<td></td>
<td>B (60%)</td>
<td>4</td>
<td>83%</td>
<td>Thiosulphate</td>
</tr>
<tr>
<td></td>
<td>C (72%)</td>
<td>2</td>
<td>83%</td>
<td>Thiosulphate UV</td>
</tr>
<tr>
<td>17 initial polysaccharide</td>
<td>D1 (60%)</td>
<td>2</td>
<td>-</td>
<td>Thiosulphate UV</td>
</tr>
<tr>
<td></td>
<td>D2 (55%)</td>
<td>4</td>
<td>-</td>
<td>Thiosulphate</td>
</tr>
<tr>
<td></td>
<td>D3 (12%)</td>
<td>4</td>
<td>-</td>
<td>Thiosulphate</td>
</tr>
<tr>
<td>18 Deacetylated polysaccharide</td>
<td>E1 (60%) in duplicate</td>
<td>6</td>
<td>55%</td>
<td>Thiosulphate UV</td>
</tr>
<tr>
<td></td>
<td>E2</td>
<td>2</td>
<td>88%</td>
<td>Thiosulphate Resin/UV Arsenite UV</td>
</tr>
<tr>
<td>19 COCH-reduced residual polymer</td>
<td>G1 (37.7%)</td>
<td>2</td>
<td>-</td>
<td>UV</td>
</tr>
<tr>
<td></td>
<td>G2 (53.5%)</td>
<td>2</td>
<td>-</td>
<td>UV</td>
</tr>
</tbody>
</table>
Thiosulphate Titration

Periodate oxidation curve of initial S19 polysaccharide.

Expt. 16
Thiosulphate Titration

Periodate oxidation curve of initial 519 polyaccharide.

Expt. 17
Periodate oxidation curve of deacetylated S19 polysaccharide.

Expt. 18
Periodate oxidation curve of carboxyl-reduced polysaccharide.
Expt. 19
Periodate oxidation studies of the polysaccharide gave conflicting results for the quantitative uptake of periodate. It proved impossible to obtain reproducible results for the amount of periodate reduced either by the spectroscopic method of Aspinall and Ferrier, or by thiosulphate titration or by arsenite titration. The results varied from 0.5 - 1.5 mole of periodate reduced per anhydro unit (see graphs p.947). Oxidation of 3 aliquots of polysaccharide and reduction of the derived aldehydes were carried out under identical conditions (expt. 16), except that in the oxidation 2 experiments were allowed to proceed for 100 h and the third for only 50 h. In all 3 experiments the carbohydrate content of the derived polyalcohols was 83% (table 9), indicating that oxidation had proceeded in the same way in all 3 experiments. However, in the 100 h oxidations recovery of polyalcohol was only 60% of the starting material whereas in the 50 h oxidation a 72% recovery was obtained, indicating in the former cases that at least 12% of overoxidation had occurred.

Analysis of the hydrolysates of the polyalcohols revealed their essential similarity. In addition to the unoxidised glucose, galactose, mannose and glucuronic acid, glycerol, erythritol and erythronic acid could be detected. The presence of the last three demonstrated that some end group and/or 1,2-linked hexose, some 1,4-linked glucose and some 1,4-linked glucuronic acid units respectively had been oxidised. The mannose which occurs at branch points and the 1,3-linked galactose would both be immune to periodate oxidation. The polysaccharide contains one acetyl group to every two units and this has been shown to be linked to the glucose at C-2 and/or C-3. Glucose comprises 3 in every 6.6 units of the polysaccharide, and therefore a high proportion of the glucose units are esterified by acetyl groups at positions which renders these units immune to periodate attack.
The polyalcohol derived from the 50 h oxidation contained 21% of uncleaved uronic acid. This is probably due to the formation of hemiacetal linkages between adjacent cleaved and uncleaved residues which hinder further oxidation (see Fig. 2). Reduction destroys these linkages and at the same time, due to the alkaline conditions, probably removes some of the acetyl groups. Thus a second periodate oxidation should result in further oxidation of the polyalcohol. Three sequential oxidations of the polysaccharide (expt. 17) showed this to be so (Table 9). Hydrolysis of the second polyalcohol (D2) showed a smaller proportion of glucose and a higher proportion of erythritol and glycerol. Methylation of this polyol confirmed this: all the end group and 1,2-linked hexose had been cleaved although a considerable quantity of 1,4-linked glucose remained. However after a third oxidation and reduction, during which a considerable quantity of material was lost, probably as oligosaccharides during dialysis, the derived polyalcohol (D3) was found to be devoid of uncleaved glucose and glucuronic acid.

Fig. 2

Diagram showing the structure of the polyalcohol.
Periodate oxidation of the carboxyl-reduced partly hydrolysed polysaccharide for 55 h (expt. 19) gave a low recovery (Table 9 p. 93), 38%, of polyalcohol (G1) which contained the expected uncleaved galactose and mannose. Some uncleaved glucose was also present, but this was proportionally less than in the hydrolysates from the polyalcohols derived from the initial polysaccharide after one oxidation. Nevertheless, after a second oxidation of polyalcohol (G1) and reduction, methylation of polyalcohol (G2) showed that 1,2-linked hexose and 1,4-linked glucose were still present in the hydrolysate, and this can only be attributed to hindrance to periodate oxidation by acetal formation as described previously.

It was found (Table 9, p. 93) that the polyalcohol (E) derived from periodate oxidation of the deacetylated polysaccharide had a considerably lower (55% as compared with 83%) carbohydrate content indicating a considerably increased periodate oxidation. Comparison of the hydrolysate of this polyalcohol with that from the initial polysaccharide showed considerably less uncleaved glucose, and a higher proportion of erythritol from the deacetylated polysaccharide showing an increased oxidation of the 1,4-linked glucose residues.

In spite of what appeared to be somewhat conflicting results it can be seen that the periodate oxidation results are in essential agreement with the linkages found by methylation of the polysaccharide.

**Partial acid hydrolysis studies**

The results of trial runs of 0.25M-oxalic acid hydrolysates (expt. 20) are shown in Table 10. Paper chromatography showed that the 5h-hydrolysate gave the highest quantity of oligosaccharides. The
chromatograms also showed that glucose is hydrolysed off first, followed by galactose and mannose before any monouronic acid could be detected in the hydrolysates.

Table 10

<table>
<thead>
<tr>
<th>Time</th>
<th>1h</th>
<th>2h</th>
<th>4h</th>
<th>5h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wt. of residual polymer and its % expressed by wt. of initial polysaccharide</td>
<td>36.2 mg</td>
<td>31.5 mg</td>
<td>21.2 mg</td>
<td>19.3 mg</td>
</tr>
<tr>
<td></td>
<td>72%</td>
<td>63%</td>
<td>42%</td>
<td>39%</td>
</tr>
</tbody>
</table>

Large scale partial hydrolysis of the crude polysaccharide (4.8 g carbohydrate) carried out under the same conditions (5h) gave the results detailed in table 11.

Table 11

<table>
<thead>
<tr>
<th></th>
<th>Carbohydrate as glucose (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial crude polysaccharide</td>
<td>4.80</td>
</tr>
<tr>
<td>Residual polymer after dialysis</td>
<td>1.00</td>
</tr>
<tr>
<td>Carbohydrate in dialysate</td>
<td>0.15</td>
</tr>
<tr>
<td>Ethanol soluble fraction</td>
<td>3.37</td>
</tr>
</tbody>
</table>

The residual polymer obtained after dialysis was shown to contain 100% carbohydrate by the phenol-sulphuric acid determination. It had a uronic acid content of 23% and after acid hydrolysis gave glucuronic acid, glucose, galactose and mannose. The proportion of glucose was less than that present in the initial S19 polysaccharide. After uronic acid reduction via the carbodiimide complex, the derived neutral polymer was obtained in 70% recovery (100% carbohydrate devoid of uronic acid).
The proportion of the sugars in this neutral polymer was found by GLC to be total glucose : galactose : mannose 3.0 : 1.4 : 1.0. Since 23% of the glucose units are derived from the uronic acid, the proportion GlcUA : Glc : Gal : Man as 1.2 : 1.8 : 1.4 : 1.0 is established for the residual polymer.

After preliminary attempts at separation of the oligosaccharides, the remaining alcohol soluble fraction (2.2 g carbohydrate) was converted into the free-acid form. This resulted in some loss since only 54% (1.17 g carbohydrate) of the material was recovered. The latter was separated into neutral fraction (460 mg) and acidic fraction (710 mg) by elution down a column of Deacidite FF-IP. The neutral and acidic fractions were further separated by preparative paper chromatography and the recovery of each fraction is shown below (Table 12).

Table 12

<table>
<thead>
<tr>
<th>Neutral fraction</th>
<th>Carbohydrate (mg)</th>
<th>Acidic fraction</th>
<th>Carbohydrate (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monosaccharides</td>
<td>325</td>
<td>01</td>
<td>4.1</td>
</tr>
<tr>
<td>F1</td>
<td>26</td>
<td>02</td>
<td>25</td>
</tr>
<tr>
<td>F2</td>
<td>3</td>
<td>03</td>
<td>500</td>
</tr>
<tr>
<td>F3 (as mixture)</td>
<td>10</td>
<td>Total of above fractions</td>
<td>529.1</td>
</tr>
<tr>
<td>F4</td>
<td>4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>F5</td>
<td>7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>F6</td>
<td>8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total of above fractions</td>
<td>383</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Out of the 460 mg neutral fraction, only 383 mg is accounted for above because higher oligosaccharides (at the starting line on a paper chromatogram) were not examined, and also because each fraction had to be re-chromatographed 2-4 times to obtain a chromatographically pure sample. Similar losses were incurred with the acidic fraction, fractions between C2 and C3, for example, were present in insufficient proportion to permit investigation.

**Neutral oligosaccharides**

The neutral oligosaccharide fractions (expt. 21) are tabulated below.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>R&lt;sub&gt;Glucose&lt;/sub&gt; in solvents</th>
<th>DP</th>
<th>Components</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1</td>
<td>0.56</td>
<td>2</td>
<td>Glc ▶ Gal ▶ Man</td>
</tr>
<tr>
<td>F2</td>
<td>0.44</td>
<td>2</td>
<td>Glc : Cal = 1:1</td>
</tr>
<tr>
<td>F3</td>
<td>0.36</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>F4</td>
<td>0.25</td>
<td>3</td>
<td>Glc ▶ Gal ▶ Man</td>
</tr>
<tr>
<td>F5</td>
<td>0.14</td>
<td>5</td>
<td>Glc, Cal, Man</td>
</tr>
<tr>
<td>F6</td>
<td>0.06</td>
<td>8</td>
<td>Glc, Cal, Man</td>
</tr>
</tbody>
</table>

(a) n-butanol: ethanol: water (40:11:19)

(b) n-butanol: pyridine : water (6:4:3)

**Neutral fraction F1**

F1 (26 mg) had the same mobility as maltose in solvent [GH 5(a)iv], \([\alpha]_D +56^\circ\) of maltose \([\alpha]_D + 140.7\), a DP of 2, gave a red colour with the tetrazolium spray and a greenish blue colour with the aniline-diphenylamine spray. On hydrolysis, glucose, galactose and mannose were obtained, indicating a mixture of disaccharides. Glucose and galactose were
Confirmed by glucose and galactose oxidases respectively. Mannose was confirmed by paper electrophoresis of the hydrolysate in molybdate buffer. \( M_{\text{Mannose}} = 1.0 \).

Methylation of the reducing disaccharide mixture gave after hydrolysis:

- 2,3,4,6-tetra-O-methylglucose (major),
- 3,4,6-tri-O-methylhexose, (see p. 83)
- 2',6-tri-O-methylgalactose,
and 2,3,6-tri-O-methylglucose.

The derived disaccharides alditols after reduction with borohydride and electrophoresis in molybdate buffer showed 3 spots with mobilities:

- 3-glucosylgalactitol with \( M_{\text{glucitol}} = 0.0 \)
- 4-glucosylglucitol with \( M_{\text{glucitol}} = 0.50 \)
- 2-glucosylmannitol/glucitol with \( M_{\text{glucitol}} = 0.66 \).

These results indicated the presence of 3 disaccharide alditols, that is,

- 3-glucosylgalactitol with \( M_{\text{glucitol}} = 0.00 \)
- 4-glucosylglucitol with \( M_{\text{glucitol}} = 0.50 \)
- 2-glucosylmannitol/glucitol with \( M_{\text{glucitol}} = 0.66 \).

Further evidence for the presence of the 3 disaccharides was obtained by methylating the disaccharide alditols after borodeuteride (\( D_4 \)) reduction. GLC and GC-MS of the permethylated disaccharide alditols gave 3 peaks with mass spectra possessing the distinguishing ions of 1,3-, 1,4- and 1,2-linked disaccharide alditols respectively.
Table 14

<table>
<thead>
<tr>
<th>Retention times of permethylated disaccharide alditols relative to permethylated maltitol</th>
<th>Distinguishing ions m/e values</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>OV-225</td>
</tr>
<tr>
<td>1,3-linked</td>
<td>1.1</td>
</tr>
<tr>
<td>1,4-linked</td>
<td>1.0</td>
</tr>
<tr>
<td>1,2-linked</td>
<td>0.9</td>
</tr>
</tbody>
</table>

The specific rotation of the F1 mixture would indicate that the 3 disaccharides have α-glycosidic links.

**Neutral fraction F2**

F2 (2.1 mg) had a DP of 2.2 and gave on hydrolysis glucose and galactose in equal proportions. Reduction to the disaccharide alditol followed by electrophoresis in molybdate buffer gave a single spot (\( M_{\text{glucitol}} = 0.66 \)) indicating a 1,2-linked disaccharide alditol. Thus F2 was identified as galactosyl(1→2)glucose. It cannot be glucosyl(1→2)galactose since the galactose residues in the polymer are solely 1,3-linked.

**Neutral fraction F3**

Electrophoresis of the derived F3 alditol in molybdate buffer gave a single spot (\( M_{\text{glucitol}} = 0.66 \)) indicating a 1,2-linked disaccharide.

**Neutral fraction F4**

F4 (4.8 mg) had a DP of 2.9 and \([\alpha]_D + 24\). On partial hydrolysis, it gave glucose, galactose, mannose together with spots with the mobilities of F1, F2, F3 and unhydrolysed F4. After borohydride reduction of the trisaccharides (2 mg) and hydrolysis, the reducing sugars obtained were \( \text{Glc} \succ \text{Gal} \succ \text{Man} \).
Chromatography on tungstate impregnated paper [5\% aqueous sodium tungstate dihydrate adjusted to pH 8 with 5\text{H}_2\text{SO}_4], showed the presence of glucitol (main) and mannitol (minor) together with the reducing sugars above. Electrophoresis of the trisaccharide alditols gave 2 spots indicating the following mixture:

<table>
<thead>
<tr>
<th>Main spot</th>
<th>Glucitol</th>
<th>Minor spot</th>
<th>Mannitol</th>
</tr>
</thead>
<tbody>
<tr>
<td>H → H → 4</td>
<td>0.27</td>
<td>H → H → 2</td>
<td>0.58</td>
</tr>
</tbody>
</table>

H = Hexose.

Reduction of the trisaccharides (1.8 mg) with borohydride followed by electrophoresis afforded isolation of the spots. The main spot after partial acid hydrolysis gave equal amounts of glucose and galactose together with glucitol and a disaccharide with chromatographic mobility corresponding to the F1 fraction. Thus the main spot is a trisaccharide comprising 2 moles of glucose and 1 mole of galactose with glucose as the reducing end unit linked at C-4.

Insufficient amount of the minor trisaccharide alditol was obtained for further study. To account for the proportion of glucose and the presence of mannose after reduction the minor trisaccharide is composed of 2 moles of mannose and one of glucose, one of the former units being the reducing end group linked at C-2.

Neutral fractions F5 and F6

F5 (6.7 mg) and F6 (7 mg) had DP of 5 and 8 respectively. On partial acid hydrolysis, they each yielded the fractions F1, F2, F3, F4 and F5 together with the monosaccharides Glucose > Galactose > Mannose.
Acidic oligosaccharides

Acidic fraction G1

G1 (4.1 mg) (expt. 22) had the same mobility as glucuronic acid on paper chromatograms, and on electrophoresis gave two spots with \( M_{Glc UA} = 0.97 \) and 1.0. It had a DP of 1.9. It was resistant to formic acid hydrolysis, and glucose oxidase showed the absence of glucose in the hydrolysate.

After reduction followed by esterification and reduction, the product contained glucitol (as shown by tungstate paper chromatography) and no reducing carbohydrate. The same product, after hydrolysis, gave glucose and more glucitol. Thus G1 must be a mixture of glucuronic acid and diglucuronic acid (major).

Acidic fraction G2

G2 (25 mg) has chromatographic mobility \( R_{Glc UA} = 0.4 \) in solvent \([GM 5(a) i]\), DP of 2.2, \([\alpha]_D^{25} = -17.2^0 \) (cf. \( \beta-D-Glc UA(\rightarrow)2Man \) of \([\alpha]_D^{28} = -28.0^0 \)), and electrophoretic mobility \( M_{Glc UA} = 0.57 \) in pyridine/acetic acid buffer (pH 6.7).

Hydrolysis of G2 gave unhydrolysed starting material together with glucuronic acid and mannose. Hydrolysis of the derived neutral reducing disaccharide gave glucose and mannose in equimolar proportion. Examination of the derived permethylated neutral disaccharide alditol gave a single peak on GLC (T = 0.95 on OV-17) and its mass spectrum after GC-MS gave the distinguishing ions of a 1,4-linked disaccharide alditol (similar to permethylated maltitol).

Examination of the hydrolysed methylated disaccharide as methylated alditol acetates gave two peaks corresponding to 2,3,4,6-tetra-O-methyl glucose and 2,3,6-tri-O-methylmannose clearly distinguishable from 3,4,6-tri-O-methylmannose. Thus G2 is the aldobiouronic acid, \( \beta-D-Glc UA(\rightarrow)4D-Man \).
Acidic fraction G3

G3 (500 mg) had an average degree of polymerisation equal to 13, and electrophoretic mobility \( M_{\text{Glc UA}} = 0.3 \) in pyridine/acetic acid buffer (pH 6.7). After partial hydrolysis, G3 yielded the neutral fractions F1 (major), F2 \( \rightarrow \) F6 together with acidic fractions G1 (containing both mono- and di-uronic acids) and G2 (major) as well as unhydrolysed starting material.

Table of oligosaccharides.

Table 15

<table>
<thead>
<tr>
<th></th>
<th>Oligosaccharide structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1</td>
<td>D-Glc(( \rightarrow ) 4)Glc.</td>
</tr>
<tr>
<td></td>
<td>D-Glc(( \rightarrow ) 2)Man/Glc.</td>
</tr>
<tr>
<td></td>
<td>D-Glc(( \rightarrow ) 3)Gal.</td>
</tr>
<tr>
<td>F2</td>
<td>Gal(( \rightarrow ) 2)Glc.</td>
</tr>
<tr>
<td>F4 (major)</td>
<td>Glc(( \rightarrow ) 3)Gal(( \rightarrow ) 4)Glc or Gal(( \rightarrow ) 2/4)Glc(( \rightarrow ) 4)Glc.</td>
</tr>
<tr>
<td></td>
<td>Glc(( \rightarrow ) 2/4)Man(( \rightarrow ) 2)Man or Man(( \rightarrow ) 2/4)Glc(( \rightarrow ) 2)Man.</td>
</tr>
<tr>
<td>G1</td>
<td>Glc UA(( \rightarrow ) 4)Glc UA</td>
</tr>
<tr>
<td>G2</td>
<td>( \beta )-D-Glc UA(( \rightarrow ) 4)Man.</td>
</tr>
</tbody>
</table>

The partial hydrolysis results are in full agreement with the linkages found from the earlier experiments. Neither glucose nor galactose appear to be present in glucuronosyl linkage in the polysaccharide. The isolation and characterisation of the aldobiouronic acid, \( \beta \)-D-Glc UA (\( \rightarrow \) 4)D-man and of the diglucuronic acid suggests a repeating unit of Glc UA(\( \rightarrow \) 4)Glc UA(\( \rightarrow \) 4)Man. There are 1.6 moles of glucuronic acid to 1 mole of mannose in S19 polysaccharide. Based upon the above
repeating unit this leaves 0.2 mole of mannose in glycosidic linkage. This could account for the release of free mannose under comparatively mild hydrolysis conditions and before any uronic acid is released, and for the presence of glucosyl(1→2)mannose in the partial hydrolysate.

The high proportion of di-O-methylmannose in the hydrolysate of the methylated polysaccharide (Table 7 p.83) means that it is the mannose in the above suggested repeating unit which occurs at the branch points.

The isolation of Gal(1→2)Glc shows that at least some of the 3,4,6-tri-O-methyl hexose is a glucose derivative (see page 83).

Although the ratio of the peak areas of 2,4,6-tri-O-methylgalactose to 3,6-di-O-methylmannose (1:1, Table 7) is the same as that found in the initial material, the total ratio of the peak areas of the tetra-, 2,3,6-tri- and 3,4,6-trimethyl sugars (that is 3:8) is considerably greater than the molar ratio of 3.0 for glucose found for the initial polysaccharide. It seems therefore that the 3,4,6-tri-O-methyl derivative is a mixture of glucose and mannose derivatives.

The fact that the fractions F5, F6 and G3 (DP, 5, 8, and 13 respectively) gave on hydrolysis the fractions F1 to F4 shows that S19 polysaccharide is built upon a regular arrangement of these different units.

Since the ratio of GlcUA:GlcMan:Gal is 1.6:3.0:1:1, if there is a repeating structure it would have a DP of at least 13. It is interesting to note that the acidic fraction G3 had an average DP of 13.
Conclusion

The extracellular polysaccharide produced by the bacterium, Xanthomonas S19, has been shown to be essentially a single polydisperse acidic heteropolysaccharide. It contains D-glucuronic acid, D-glucose, D-galactose and D-mannose in the approximate molar ratio of 1.6:3:1:1. Acetate esters, located on C-2 and/or C-3 positions of the glucose units, account for a degree of acetylation equal to 2. The linkages of the sugar units found by methylation are detailed below (Table 16). These are in agreement with the results obtained from periodate oxidation and partial hydrolysis.

This polysaccharide is similar in some respects to other Xanthomonas extracellular polysaccharides, but it also has some major structural differences. Glucose is linked to C-3 of the galactose residues in S19 polysaccharide as well as in X. stewartii polysaccharide. Glucose is also linked to C-4 of glucose residues in S19 polysaccharide as well as in X. campestris and X. hyacinthi polysaccharides.

The 1,4-linkage of the glucuronic acid found in S19 polysaccharide is common to the extracellular polysaccharides of X. campestris and of X. oryzae. Mannose is found in glucuronosyl linkage in S19 polysaccharide as well as in the above polysaccharides and also in that from X. hyacinthi. However, it differs in that in S19 polysaccharide, the uronic acid is linked to C-4 of the mannose, instead of to C-2 as found for the other Xanthomonas polysaccharides, where no 1,4-linked mannose is present. Both galactose and mannose are present in the S19 polysaccharide whereas other Xanthomonas polysaccharides examined contain either mannose or galactose together with the common constituents.
glucose and glucuronic acid. Pyruvic acid has been reported in 4 Xanthomonas species (see p.24) but acetate has so far been found only in X. campestris polysaccharide.

Table 16. Comparison of linkages

<table>
<thead>
<tr>
<th></th>
<th>Glucose</th>
<th>Galactose</th>
<th>Mannose</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>end-group</td>
<td>1,3-</td>
<td>end-group</td>
</tr>
<tr>
<td>Xanthomonas</td>
<td>1,4-</td>
<td>1,6-</td>
<td>1,2-</td>
</tr>
<tr>
<td>polysaccharides</td>
<td>1,6-</td>
<td>1,3,4,6-</td>
<td>1,2-</td>
</tr>
<tr>
<td></td>
<td>1,2,4-</td>
<td>1,3,4-</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1,3,4-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>S19 polysaccharide</td>
<td>end-group</td>
<td>1,3-</td>
<td>1,2- (minor)</td>
</tr>
<tr>
<td></td>
<td>1,2-</td>
<td></td>
<td>1,2,4- (major)</td>
</tr>
</tbody>
</table>
PART TWO

Structural investigation of the extracellular polysaccharide metabolised by the unicellular red alga, Rhodella maculata.
INTRODUCTION

The algae of the sea have a great variety of forms ranging from tiny unicellular to giant multicellular photosynthetic organisms. According to the pigments they contain, they can be classified into four (4) main groups, namely:

1. Phaeophyceae (brown)
2. Rhodophyceae (red)
3. Chlorophyceae (green)
4. Cyanophyceae (blue-green)

Rhodella maculata belongs to the second group and the characteristic polysaccharides of this group will be discussed.

Polysaccharides of the red seaweeds

A. Floridean starch

This is the food reserve of the Rhodophyceae and it stains red-violet with iodine. It is a homopolymer of glucose and closely resembles the amylopectin of higher plants in its structure and many of its properties. The main chain is comprised of $\alpha-1,4$-linked glucose units. There is tentative evidence that this is interspersed with a few $\alpha-1,3$-linkages in some samples. Branch points are due to $\alpha-1,6$-linkages. The main difference between higher plant starches and floridean starch seems to be the shorter average chain length of the latter.

B. Mannan

The mannans from Porphyra umbilicalis, for example, is a linear polymer of $\beta-1,4$-linked mannose units, with an average chain length of
about 12. It occurs as an extracellular skeletal cuticle and is insoluble in water.

C. **Cellulose**

There is very little detailed structural information on the red algal cellulose. The \( \alpha \)-cellulose\(^{228} \) of *Gelidium amansii* resembles cotton cellulose.

D. **Xylans**

Xylans from red algae fall into two types:

I. Water soluble xylans: These are not skeletal material and are most likely food reserve polysaccharide. They consist of both \( \beta-1,3- \) and \( \beta-1,4- \) xylosidic linkages together in a structure that may be branched or linear. These structures may be either a pure xylan or a heteropolysaccharide.

II. Cell-wall material: These xylans are essentially linear and consist of either \( \beta-1,3- \) linked or \( \beta-1,4- \) linked xylose residues.

*Rhodymenia palmata* exhibits both types of xylans. Its water soluble xylans\(^{229} \) consist of essentially linear chains of \( \beta-1,4- \) linked (80–84\%) and \( \beta-1,3- \) linked (16–20\%) xylose units. These units are randomly arranged in the macromolecule. Some heterogeneity\(^{230} \) has been demonstrated in these polysaccharides. The other type is essentially a linear \( \beta-1,4- \) linked xylan.\(^{231} \)

*Porphyra umbilicalis*\(^ {231} \) also contains both types of xylan: one being a linear \( \beta-1,3- \) linked xylan,\(^{232} \) and the other consisting of 1,4-linked (77\%) and 1,3-linked (23\%) xylose units in the polymer.
Rhodochorton floridulum possesses a branched xylan, possibly in a heterostructure which contains 1,3-linked glucose units. The branch points (about 10%) are located in 1,3,4-linked xylose units.

E. Galactans

The galactans, being the major polysaccharides, are characteristic of Rhodophyceae, and contain varying proportions of D- and L-galactose and 3,6-anhydro D- and L-galactose, which may carry O-methyl groups and ester sulphate groups. They are typified by 3 main groups, that is, agar-, porphyran-, and carrageenan-like polysaccharides. There are similarities within these groups and other galactans may have intermediate characteristics. A common feature is the presence of chains made up of alternating 1,3-linked galactoses or monomethylgalactoses and 1,4-linked 3,6-anhydrogalactoses, for example, as in agarose (see Fig. 3).

F. Other polysaccharides

Two Grateloupia species, namely Aeodes orbitosa and Phyllymenia cornea produce sulphated galactans which consist of equal proportions of 1,3- and 1,4-linkages. Some of these occur as alternating units as with other galactans of the Rhodophyceae.

The extracellular polysaccharides of the unicellular microscopic red algae Porphyridium aerugineum and Porphyridium cruentum contain in common xylose, galactose, glucose and uronic acid in different proportions, together with an unidentified constituent.

The present thesis describes an investigation of the extracellular mucilage of the unicellular microscopic red alga, Rhodella maculata.
\( X = \)

\[
\begin{align*}
X & = \\
& = \end{align*}
\]

\( Y = \)

\[
\begin{align*}
Y & = \\
& = \end{align*}
\]

\( R = H \text{ or } CH_3 \)

Fig. 3
EXPERIMENTAL

Expt. 1. Growth of Rhodella maculata

The organism, *Rhodella maculata*, was cultured at the University of Leeds by L.V. Evans and M. Callow. The cells were grown in either SWM$_3$ medium or ASP12 medium. In experiments where the amount of sulphate supplied was varied ASP12 was used, MgSO$_4$ being replaced by MgCl$_2$. Cultures of 100 ml in 250 ml flasks were kept in suspension by gentle shaking and illuminated by 4000 lux from Northlight fluorescent tubes with a 6 h photoperiod.

Expt. 2. Isolation of the mucilage

Cultures were centrifuged at 10,000 g for 15 min to pellet the cells. The supernatant was passed through 1-μm Millipore filters, and dialysed for 48 h against running tap water and 24 h (2 changes) against deionised water. The polysaccharide (solubilised mucilage) was refiltered through 0.45 μm Millipore filters and freeze-dried to a white solid (yield 200 mg/L of culture medium after 20 days) or precipitated with absolute ethanol (4 vol) and recovered by centrifugation at 40,000 g for 30 min. Mucilage associated with the pellet cells (encapsulating mucilage) was removed by shaking with water at 40°C for 48 h. After centrifugation at 50,000 g for 30 min, the supernatant was filtered, dialysed and recovered as above.

In some experiments, the supernatant was not filtered, but only dialysed as above and freeze-dried.

Mucilage obtained from cultures containing sulphate is labelled S$^+$ and mucilage obtained from sulphate-free medium is called S$^-$. 
Expt. 3. Preliminary examination

Aliquots of each of the batches (see p.128) of mucilage were tested for carbohydrate content and were subjected to acid hydrolysis [GM 3a] and the hydrolysates analysed by paper chromatography (solvents [GM 5(a) i, ii] and sprays [GM 5(b) i-iii]).

Expt. 4. Determination of uronic acid, sulphate and ash contents of the polysaccharide

$S^+$ and $S^-$ mucilages were each separately analysed for uronic acid [GM 2(b) i, ii] and for ester sulphate [GM 2e]. The ash contents of $S^+$ mucilages were also determined and the sulphate content of the ash estimated in one experiment.

Expt. 5. (a) Fractionation on DEAE-cellulose column.

Preparation of column: Diethylaminoethyl cellulose (microgranular, DE52, fresh) (12 g) was degassed in deionised water (75 ml) (pH 5.5-6.0) for 2 h. The slurry was poured down a 100 ml cylinder, and as soon as a supernatant was formed, it was removed. In this way, fines were effectively removed. The resulting slurry was poured down a glass column, 0.9 cm x 16 cm = 10 cc capacity. The column was washed with deionised water (30-40 ml) and it was then ready for use.

Chromatography: The mucilage $S^+, (1.2$ mg) in water (2 ml) as a homogeneous gel was applied to the column. Neutral fractions were collected by elution with water (pH 5.5-6.0): the first 40 ml of eluant gave fraction 1 and the following 200 ml gave fraction 2. An acid fraction was collected by elution with 0.3M-KCl.

The neutral fractions were each concentrated to dryness and hydrolysed with 90% formic acid (0.75 ml) [CM 3a]. The hydrolysates were examined by paper chromatography [CM 5(a) i and 5(b) i].
The acid fraction was dialysed against water until free from KCl, concentrated to dryness, hydrolysed with formic acid [CM 3a] and examined on paper [CM 5(a)i,ii and CM 5(b)i].

(b) Ultracentrifugation
The S+ mucilage (0.3% wt/wt solution in 0.1M NaCl) was analysed in a 1 ml cell on a Beckman Spinco Model E ultracentrifuge at 60,000 r.p.m. A 0.15% solution in 0.1M NaCl was also examined in a 2 ml cell at 20,000 r.p.m. and 33,000 r.p.m.

Expt. 6. Identification of the constituents
(a) By paper chromatography: S+ mucilage (15 mg), S- mucilage (5 mg) and desulphated S+ mucilage (see expt. 9) (5 mg) were hydrolysed separately with formic acid [CM 3a], and an aliquot of each hydrolysate was analysed by paper chromatography solvents [CM 5(a)i-iv, ix] and sprays [CM 5(b) i-vi, ix]). In addition to spots with the mobility of xylose, glucuronic acid (major), galactose, glucose and rhamnose (minor), and in some samples fructose and/or mannose (trace) all the hydrolysates gave a spot of high mobility which stained pink with aniline oxalate. In an attempt to identify this and confirm the rhamnose the following standards were run on paper against the hydrolysates of the mucilage: acidic hydrolysate of agar, carrageenan, and apiin; 4-0- and 6-0-methylgalactose, ribose, lyxose, 6-deoxyglucose, 2-, 3- and 4-0-methylxylose and 2- and 3-0-methylfucose.

(b) By GLC: TMS derivatives of the hydrolysate of S+ mucilage as the aldoses and alditols were examined by GLC [CM 5(f)(i) v] and the retention times compared with those of above standards.
(c) The material with the same mobility as that of rhamnose and that of $R_{gal} 2.2$ [Butanol: ethanol: water 40:11:19] were separated from a hydrolysate by preparative paper chromatography. Both substances were examined by GLC as their TMS derivatives before and after reduction to their respective alditols, and the retention times compared with those of rhamnose and 3-0-methylxylose run as controls. The identity of the suspected 3-0-methylxylose was confirmed by conversion into the aldose and alditol acetate derivatives and analysis by GLC on columns of 3% OV-225 and 3% OV-17 [GM 5(f)(ii)a,f] and by GC-MS [GM 5g]. Conversion into the alditol was with sodium borodeuteride ($d_4$). The substance was also subjected to demethylation [GM 4j] with boron trichloride. A parallel experiment was carried out on 2-0-methylfucose. The products were each examined on paper [GM 5(a)i,ii and GM 5(b)ii].

(d) After reduction of the uronic acid:- A further aliquot of $S^+$ mucilage hydrolysate was esterified [GM 4d] and the product reduced with sodium borohydride. The derived neutral material was divided into 2 parts. One part was hydrolysed with formic acid [GM 3a] and the other part with 0.5M-$H_2SO_4$ [GM 3(b)i] and both were analysed by paper chromatography and by GLC as under (a) and (b).

(e) By electrophoresis:- The hydrolysates of the mucilage were also subjected to electrophoresis in pyridine/acetic acid buffer [GM 5(d)iv].

(f) Confirmation of the presence of protein:- The $S^+$ mucilage was hydrolysed with 12M-$HCl$ [GM 2i] and the hydrolysate examined on an amino-acid analyser.
Expt. 7. Determination of the ratios of the constituents

(a) The ratio of glucose to galactose was determined with glucose and galactose oxidases on a hydrolysate of S⁺ mucilage (see expt. 3) both before and after esterification, reduction and re-hydrolysis.

(b) The hydrolysate from S⁺ mucilage was applied to a chromatography paper and eluted in solvent [CM 5(a)ii] for 20 h. The positions of the different constituents was located by spraying sides strips. Each was then eluted and the eluate filtered through glass fibre paper, and the amount of sugar present determined by phenol sulphuric acid [CM 2a], the absorption being read off the appropriate standard graphs for each sugar.

Expt. 8. Viscosity Determinations

Viscosity measurements were made in an Ostwald viscometer (No. 2 BSS). The highest concentration of mucilage that could be used was 0.35% by weight. Measurements were carried out on this and on a 0.1% solution at 25ºC for both these solutions and also at 70ºC for the latter solution. Both solutions were autoclaved at 115ºC for 20 min and their viscosities redetermined.

Expt. 9. Desulphation of the mucilage

The polysaccharide (S⁺, 31.6 mg) in water (20 ml) was treated with potassium borohydride (19 mg) with shaking for 20 h at room temperature. Additional borohydride (30 mg) and sodium hydroxide (10 ml, 3N) were added and the mixture heated for 8 h at 80ºC. The cooled mixture was set aside overnight at room temperature and the solution dialysed. The material (24 mg) remaining in the dialysis sac was recovered by freeze-drying, and analysed for sulphate (expt. 4).
The dialysates (1800 ml) were concentrated, after neutralisation, to 12 ml and the carbohydrate content determined. Cations were removed with Amberlite IR-120(H+) resin and the resulting solution was assayed for sulphate. This was also examined by paper chromatography [CM 5(a)ii]. Both silver nitrate and aniline oxalate were used as locating agents.

Thiobarbituric acid tests [CM 2j] were carried out both on the dialysate and on the freeze-dried material from the dialysis sac.

**Expt. 10. (a) Presence of 3,6-anhydrogalactose**

The mucilage (9.8 mg) was hydrolysed with N-H2SO4 (2 ml) for 2 h at 70°C. The partially hydrolysed polymer, after cooling, was precipitated with ethanol (6 vol.). After centrifugation, the supernatant was neutralised with barium carbonate [CM 3b]. The filtrate was examined by paper chromatography in [CM 5(a)ii,iv] and then further hydrolysed with sulphuric acid [CM 3b] and examined on paper as above.

(b) **Assay of 3,6-anhydrogalactose**

Aliquots (2 ml) of the mucilage (S+), of the desulphated mucilage [5 mg each in water (20 ml)], and of xylose and glucuronic acid (2.5 and 0.75 mg, respectively) in water (20 ml), were treated separately with resorcinol reagent (10 ml) according to the method of Yaphe 244 and the absorptions read at 550 nm.

**Expt. 11. Infra-red spectra of the mucilages**

It was not possible to make a thin film of Rhodella mucilage because sufficient material was not available. A solution of the mucilage [S+, 3 mg and desulphated S+, 3 mg] was each evaporated to dryness in the presence of potassium chloride (7 mg). The salt
mixtures were each dried in a vacuum desiccator overnight before conversion into KCl discs. The spectra were determined as in [GM 5(h)ii].

Expt. 12. Methylation studies

(a) The mucilage (S⁺, 16.3 mg and 14 mg), 1,3-linked xylan (from Caulerpa filiformis) (15 mg), 1,3- and 1,4-linked xylan (from Rhodymenia palmata) (15 mg) and the desulphated mucilage (expt. 9) (8 mg) were each methylated by the Hakomori method [GM 4h]. The various methylated materials were hydrolysed [GM 3a] and the methylated sugars in a portion of each hydrolysate were converted into the methylglycosides [GM 4d] and examined by GLC [GM 5(f)i]. A second portion of each of the hydrolysates was converted into the methylated alditol acetates [GM 4 c,f] and examined by GLC and GC-MS [GM 5(f)(ii)α] and [GM 5g]. The sulphate content of a portion of the hydrolysate from the methylated S⁺ mucilage was also determined.

(b) Methylation, and reduction of the uronic acid:

(i) The mucilage (S⁺, 30 mg) was partially methylated with methyl iodide in Ag₂O/DMF [GM 4i] (twice), and the product was reduced with lithium aluminium hydride [GM 4n]. The derived neutral polymer was remethylated by the Hakomori method [GM 4h]. After hydrolysis, the methylated sugars were characterised as their alditol acetate.

(ii) Methylated mucilage S⁺ from (a) was reduced with lithium aluminium hydride and the derived neutral polysaccharide was remethylated by the Hakomori method. The product was hydrolysed and examined as above.

(iii) The mucilage recovered after autoclaving a 0.35% solution (expt. 8) was methylated by the Hakomori method [GM 4h] and the product was methanolysed [GM 4b]. The methanolysate was reduced with sodium
borodeuteride in water-methanol (1:1 v/v) and the derived neutral
product was hydrolysed and analysed as in (i) above.

(iv) The derived neutral polysaccharide obtained after reduction via
the carbodiimide complex (see expt. 16) was methylated by the Hakomori
method [GM 4h] and the product was hydrolysed and examined as in (i)
above.

Expt. 13. Periodate Oxidation

Mucilages ($S^+$ and $S^-$) (50 mg each in water, 50 ml) were
oxidised separately with 0.03M-sodium metaperiodate (50 ml) and the
reaction was monitored by the method of Aspinall and Ferrier. After
44 h, the reaction mixture was worked up as in [GM 4m(i)] to yield $S^+$
polyol I (30.2 mg) and $S^-$ polyol I (26.4 mg). These polyalcohols
(23.8 mg and 18.8 mg respectively) were re-oxidised with 0.015M-periodate
solution (10 ml), and worked up as before to yield $S^+$ polyol II (7.5 mg)
and $S^-$ polyol II (6.3 mg). These polyalcohols as well as $S^+$ polyol I
(6.5 mg) and $S^-$ polyol I (7.6 mg) were each hydrolysed with formic acid
[GM 3a] and examined on paper (solvents [GM 5(a) i, ii, v] and sprays
[GM 5(b) i, ii]), and by electrophoresis in pyridine/acetic acid buffer
(pH 6.7) [GM 5(d)iv].

Expt. 14. Polyacrylamide gel electrophoresis

The Shandon SAE-2734 analytical polyacrylamide electrophoresis
apparatus was used, and Shandon general instructions were followed.
Further details are described in Appendix 3 p.155. The mucilage (71 mg)
in water (5 ml) was shaken for 3 days to give a homogeneous pale brown
gelatineous solution. This (100 μl) was applied to each tube of poly-
acrylamide gel (8 tubes in all). Stacking was done by applying 1mA per
gel tube for 20 min. The run was continued for 50 min. at 30mA.
Staining was with either toluidine blue (deep blue band indicates acidic sulphated polysaccharide), or periodate-Schiff reagent (purple with polysaccharide vulnerable to periodate), or Coomassieblue (blue with protein) or "fetains-all" (pink or red with protein, and it also stains acidic polysaccharides).

Expt. 15. (a) **Examination of oligosaccharides**

Mucilage ($S^+$, 19.8 mg) was hydrolysed with formic acid [GM 3a]. The hydrolysate was separated in 3MM paper [GM 5(a) ii]. The material near the base line (oligosaccharides) was eluted and its pH tested. This was esterified, reduced with sodium borohydride in water, and hydrolysed with formic acid [GM 4g]. The hydrolysate was examined by paper chromatography and by gas-liquid chromatography.

(b) **Examination of aldobiuronic acids**

Mucilage (30 mg) was hydrolysed with formic acid [GM 3a]. The hydrolysate was separated by preparative paper chromatography on 3MM paper [GM 5(a) i]. A slow moving band ($R_{gal} = 0.5$) was eluted off separately. Its carbohydrate content was determined and it was examined by paper electrophoresis in pyridine/acetic acid buffer [GM 5(d)iv]. Its DP was estimated by the Timell method [GM 2g]. It was esterified, reduced and hydrolysed (2 h with formic acid) [GM 4g]. The hydrolysate was examined by paper chromatography and by GLC of the TMS-derivatives. Both silver nitrate and aniline oxalate were used as locating agents.

Expt. 16. **Reduction via carbodiimide**

The mucilage ($S^+$, 13.6 mg) was dissolved in water (10 ml) by shaking for 36 h. Its carbohydrate content [GM 2a] were determined.
The same batch of mucilage (S⁺, 123 mg) was homogenised in water (20 ml) and reduction of the uronic acid was carried out by the method of Taylor and Conrad. To the solution, ethyl-3-dimethylaminopropyl carbodiimide hydrochloride (1 g EDC) was added and the pH brought to pH 4.7 with a few drops of 0.1M-HCl. This pH was maintained for 2h. Then sodium borohydride (7.6 g/100 ml) was added at first drop by drop, and then 0.2 ml at a time while the pH was maintained below 7, preferably between pH 6.5-7.0 with 4M-HCl. This process took about 3 h. The solution was poured into ethanol (6 vol) and the precipitated polysaccharide was recovered by centrifugation. This was further dialysed against water. The aqueous polysaccharide solution was concentrated to 10 ml, its carbohydrate content [GM 2a] and uronic acid content [GM 2(b)iii] were determined.

EDC (1 gm) was added to the polysaccharide solution and the above procedure repeated, omitting the alcohol precipitation.
RESULTS AND DISCUSSION

The extracellular mucilage from Rhodella maculata, freeze-dried to a white fibrous solid, consists mainly of polysaccharide. It is soluble in water only in dilute solutions and appears to have a low positive specific rotation. Mucilage solutions in concentration as low as 0.3% are viscous and opaque. The following tables show the viscosities of two solutions before and after autoclaving.

Table 17

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Temperature</th>
<th>Viscosity (Seconds)</th>
<th>Relative Viscosity</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1% by wt. Rhodella mucilage</td>
<td>25°C</td>
<td>17.5</td>
<td>1.0</td>
</tr>
<tr>
<td>[44% carbohydrate content (Glc UA: Xyl 1:4 graph), see p.135]</td>
<td>61</td>
<td>3.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>70°C</td>
<td>31</td>
<td>1.8</td>
</tr>
<tr>
<td></td>
<td>25°C after first at 70°C</td>
<td>61</td>
<td>3.5</td>
</tr>
<tr>
<td></td>
<td>after autoclaving, at 25°C</td>
<td>50</td>
<td>2.9</td>
</tr>
</tbody>
</table>

Heating this solution to 70°C appeared to cause no degradation and even autoclaving did not appear to cause excessive degradation. In contrast autoclaving a more concentrated solution appeared to cause an appreciable amount of depolymerisation.

Table 18

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Time</th>
<th>Relative Viscosity</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.35% by wt. mucilage solution (pH = 6)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Carb. content = 57% before and after autoclaving</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Solution at 25°C</td>
<td>4 h</td>
<td>823</td>
</tr>
<tr>
<td>After autoclaving, solution at 25°C</td>
<td>0.5 h</td>
<td>103</td>
</tr>
<tr>
<td>solution at 70°C</td>
<td>607 sec</td>
<td>35</td>
</tr>
</tbody>
</table>
The carbohydrate, nitrogen and ash content of different batches of mucilage supplied are given below:—

Table 19

<table>
<thead>
<tr>
<th>Date supplied</th>
<th>Weight of mucilage (mg)</th>
<th>Carbohydrate content*(%)</th>
<th>Ash content (%)</th>
<th>Protein (%) (from nitrogen content)</th>
</tr>
</thead>
<tbody>
<tr>
<td>June 1973</td>
<td>69</td>
<td>62.5</td>
<td></td>
<td>16</td>
</tr>
<tr>
<td>Aug. 1973</td>
<td>59</td>
<td>36.5</td>
<td></td>
<td>16</td>
</tr>
<tr>
<td>Dec. 1973</td>
<td>57</td>
<td>45.5</td>
<td></td>
<td>16</td>
</tr>
<tr>
<td>June 1974</td>
<td>74(5)</td>
<td>79</td>
<td></td>
<td>16</td>
</tr>
<tr>
<td>Sept. 1974</td>
<td>53.5</td>
<td>18.6</td>
<td></td>
<td>16</td>
</tr>
<tr>
<td>July</td>
<td>80</td>
<td>n.d.</td>
<td></td>
<td>16</td>
</tr>
<tr>
<td>Aug. 1974</td>
<td>31.5</td>
<td>n.d.</td>
<td></td>
<td>16</td>
</tr>
<tr>
<td>Sept. 1974</td>
<td>46.8</td>
<td>n.d.</td>
<td></td>
<td>16</td>
</tr>
<tr>
<td>Jan. 1975</td>
<td>62.6</td>
<td>57</td>
<td></td>
<td>16</td>
</tr>
<tr>
<td>April 1975</td>
<td>500</td>
<td>46.5</td>
<td></td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>400</td>
<td>37</td>
<td>16</td>
<td>26</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>23</td>
<td>25</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>45</td>
<td>5.6</td>
<td>22</td>
</tr>
</tbody>
</table>

* Read off a glucuronic acid:xylose 1:4 graph (see p.135).

n.d. = not determined.

The wide differences in the carbohydrate content can be accounted for by the different protein and inorganic salts contents. The latter is surprising since all the batches had been dialysed. However, hydrolysates of the different samples revealed only minor qualitative and
quantitative differences, such as the presence and absence of trace quantities of mannose and fructose, in their constituent sugars. In every sample xylose and glucuronic acid were the major constituents with smaller amounts of glucose and galactose and two spots of higher mobility.

Attempts to fractionate the mucilage on DEAE-cellulose (expt. 5a) failed to yield any neutral polysaccharide in the aqueous eluate. Elution with potassium chloride gave unchanged acidic polysaccharide. The relative insolubility and gelling properties of the mucilage prevented the formation of a true boundary peak on the analytical ultracentrifuge (expt. 5b).

Gel electrophoresis (expt. 14) on the mucilage gave several bands when the gels were stained with toluidine blue for acidic polysaccharide, with periodate-Schiff reagent for polysaccharide, with stains—all \[1\text{-ethyl-2-3-(1\text{-ethyl-naphtho 1,2d thiazolin-2-ylidene)-2-methyl-
propenyl-naphtho 1,2d thiazolium bromide}\], and with Coomassie blue for protein (see Fig. 4, p. 130). It can be seen that there is a main band due to polysaccharide. This was brown before staining, dark blue with toluidine blue, purple with periodate-Schiff reagent and blue with stains—all. It does not however show up with Coomassie blue showing that is free from protein. A second band is also visible with toluidine blue but does not appear with periodate-Schiff reagent. This also gives a positive reaction with Coomassie blue indicating that it also contains protein. This may be regarded as tentative evidence for the presence of glycoprotein.

Constituents of the mucilage and their characterisation

When the alga was cultured in the presence of inorganic sulphate the extracellular mucilage had a sulphate content of 10.3% by weight which
1. **NO STAINING**

   - Running gel
   - end of gel
   - stacking gel
   - brown band

2. **TOLUIDINE BLUE**

   - dark blue
   - faint blue
   - dark blue

3. **PAS REAGENT**

   - slight purple colour
   - sharp purple

4. **"STAINS-ALL"**

   - faint blue
   - pink
   - bluish green
   - purple
   - blue

5. **COOMASIE BLUE**

   - light blue
   - blue

*Fig. 4*
corresponds to 16.5% based on the carbohydrate content, that is one sulphate group to approximately every three anhydro sugar units. In contrast the mucilage was devoid of sulphate when cultured in the absence of inorganic sulphate.

Sugars corresponding to each of the spots on the paper chromatogram of a hydrolysate were separated and characterised. The identity of the xylose, glucose and galactose was each confirmed by the retention times of their TMS derivatives on a gas liquid-chromatograph both before and after reduction to the corresponding alditol. The glucose and galactose were confirmed as the D-sugars by D-glucose and D-galactose oxidases respectively, and the content of these two sugars in the hydrolysate was estimated by the use of these two enzymes to be about 1% and 3% respectively. The retention time of xylose penta-acetate on GLC also confirmed its identity and its $[\alpha]_D + 13^\circ$ [cf. D-xylose + 18^\circ] proved it to be the D-sugar.

The material from the first unknown spot had the mobility and colour reactions of rhamnose on paper chromatograms. However in view of the fact that many red algal polysaccharides contain monomethylgalactoses which behave similarly to rhamnose on paper chromatography (see appendix p.153) and also that rhamnose has never been reported as a constituent of Rhodophycean polysaccharides it was decided, in spite of the small quantity present, to separate it and to characterise it as rhamnose. This was done as for xylose, apart from measurement of the rotation (see appendix p.154). The retention times on GLC distinguished it from the methylated galactoses and this was further confirmed in the methylation studies described later.
The substance, $R_{gal} 2.2$, corresponding to the second unknown spot on a paper chromatogram was found to have an identical mobility with that of 3-0-methylxylose and the same retention time as the TMS-derivative on GLC before and after reduction (see appendix 2, p. 154). Its identity was further confirmed by the retention times of the acetyl derivatives on GLC and by the fragmentation pattern of a mass spectrum of the alditol acetate deuterium labelled at C-1 (see mass spectrum, p. 133). After demethylation, xylose was the only sugar which could be detected. This is the first time 3-0-methylxylose has been reported as a constituent of any polysaccharide.

The presence of uronic acid was indicated by a positive naphthoresorcinol test. Only trace quantities of monouronic acid could be detected on paper chromatograms of hydrolysates and these were insufficient for characterisation. However, it appeared to be glucuronic acid since the proportion of glucose was considerably increased after esterification and reduction of a hydrolysate of the polysaccharide (expt. 61). The presence of galacturonic acid is not completely eliminated although unlikely since the ratio of glucose to galactose before esterification and reduction was 1:3 and after reduction 3:1. Some loss is to be expected during these operations but it seems unlikely that galacturonic acid is a constituent of the mucilage.

Determination of the amount of uronic acid by the carbazole method gave values of 10-12% by weight and 22-27% based on the carbohydrate content, and by the metahydroxydiphenyl method 12% by weight and 27% based on carbohydrate. These figures can only be regarded as approximate in view of the variation in carbohydrate content of the various samples of mucilage.
1,2,4,5-tetra-O-acetyl-3-O-methyllysitol-1-2H.

Paper chromatography of the hydrolysate in solvent: water saturated ethylmethylketone containing 3% cetylpyridinium chloride, confirmed the absence of any sulphated sugars. This solvent increases the mobility of sugar sulphates by as much as 10-fold. All the hydrolysates contained oligouronic acids, and amino acids were also detected. Analysis, on an amino acid analyser, of a hydrolysate obtained with 13H-hydrochloric acid gave the following amino acids:-- lysine, histidine, arginine, aspartic acid, threonine, serine, glutamic acid, proline, glycine, alanine, valine, isoleucine, leucine, tyrosine and phenylalanine.

**The presence of 3,6-anhydrogalactose**

The standard method of assay for 3,6-anhydrogalactose, a constituent of red algal galactans, is the production of a deep pink colour with resorcinol reagent, which absorbs at 550 nm. It was found that the mucilage gave the deep pink colour with the reagent. At the same time it was also observed that with the resorcinol reagent both xylose and glucuronic acid gave a bluish mauve colour, which also absorbed at 550 nm. To determine the amount of 3,6-anhydrogalactose in the mucilage it was therefore necessary to measure the absorption of a solution containing the quantity of xylose and glucuronic acid it is considered that 5 mg of mucilage would contain, and to subtract this from the absorption produced by this weight of mucilage. The absorption of xylose-glucuronic acid mixture was about 20% of that of the mucilage which was calculated to contain about 3% of 3,6-anhydrogalactose by weight. There was no increase in the amount of this anhydrosugar after alkaline desulphation.
The proportion of the constituents and estimation of the carbohydrate content of the mucilage

An accurate estimation of the proportions of the different sugars in this mucilage is difficult. The ratios of the sugars and oligo-uronic acids in a hydrolysate, after elution from a paper chromatogram, (expt. 7) were found to be xylose: 3-0-methylxylose:rhamnose:hexose: oligouronic acids = 6.5 : 2.7 : 1.6 : 1.0 : 8.0. It was impossible to separate completely the glucose and galactose and they were therefore determined together as hexose. From earlier enzymic estimations of the proportion of these two sugars in the hydrolysate the ratio is approximately 1 for glucose to 3 for galactose. At the same time it must be remembered that there is also about \( \frac{3}{6} \) 3,6-anhydrogalactose present.

From a 25% uronic acid content of the mucilage, it may be assumed that of the 8 parts of oligouronic acid 5 parts are uronic acid.

In order to determine the carbohydrate content of the mucilage by the phenol sulphuric acid method it was necessary to have a standard graph plotted from determinations on a synthetic mixture which as nearly as possible contained the proportions of sugars present in the mucilage. It is clear from the above that it was impossible to make a completely accurate mixture and it was decided therefore to measure all the carbohydrate contents from a graph constructed from a mixture of glucuronic acid (1 part by weight) and xylose (4 parts by weight). This, in the author's opinion, represents a fairly accurate measure of the carbohydrate content of the mucilage.
Desulphation of the mucilage and possible location of the sulphate residues

The sulphate, apart from about 1%, was found to be exceedingly alkali and acid labile. The desulphated polysaccharide was recovered in about 85% yield after removal of the sulphate (apart from ca. 1%) by alkali, and a paper and GLC of a hydrolysate of the desulphated material were almost identical with those of the initial polysaccharide. If the sulphate is present as an ethereal sulphate, \( R - O\overset{\text{S}}{\text{O}} - ONa, \)
then on incineration half of the sulphate should be lost since the sodium in the residue would be sufficient to produce only half a molecular proportion of \( Na_2SO_4. \)

The mucilage in the form of the sodium salt was found to give 15% of ash on incineration and analysis of the ash gave 10% of sulphate based on the weight incinerated. Unfortunately, the presence of 10% sulphate cannot be regarded as proof that the sulphate is not present as ethereal sulphate, since it is possible that sodium from the sodium uronate has combined with some of the sulphate and retained it in the ash. It does, however, seem very unlikely that the sulphate is present as cyclic sulphate, as then the polysaccharide would yield only 9.7% ash.

The failure to detect any other sugar on alkali desulphation indicates the absence of trans elimination and epoxide formation from the unmethylated 1,4-linked xylose units sulphated at C-2 or C-3. Such units on hydrolysis should yield arabinose. The presence of a methoxyl group or linkage at C-3 of the xylose units should make any sulphate on C-2 or C-4 or both stable to alkali. Furthermore the infrared pattern of this mucilage is quite different from that of other sulphated algal polysaccharides. The spectra contained no absorption
bands at a frequency of 1240 cm\(^{-1}\), characteristic of S=O stretching frequency, or at 820-850 cm\(^{-1}\), considered to be characteristic of other sulphated algal polysaccharides.\(^{233}\) However, the mucilage gave absorption bands at 1200 cm\(^{-1}\), 875 cm\(^{-1}\) and 950 cm\(^{-1}\), all of which disappeared on desulphation. It is interesting to note that the infrared spectrum of heparin, in which the iduronic is sulphated on C-2, gives absorption bands at 1230 cm\(^{-1}\), 875 cm\(^{-1}\) and 925 cm\(^{-1}\).\(^{243}\) It is tempting therefore to conclude that it is the uronic acid units in \textit{Rhodella} mucilage which are sulphated.

Although only xylose and glucuronic acid are present in sufficient quantity to accommodate all the sulphate groups it is possible that some are located on the galactose as is commonly the case in red algal polysaccharides. The presence of 3,6-anhydrogalactose leads one to expect that some of the 1,4-linked galactose residues carry sulphate on C-3 at some stage in the growth of the alga. However, there was no detectable increase in 3,6-anhydrogalactose on alkali desulphation indicating the absence of sulphate on C-3 of 1,4-linked galactose units in the present mucilage. In addition, any sulphate on C-4 or C-2 of the 1,3-linked galactose should be stable to alkali and this might account for the residual 1% sulphate.

Unlike other sulphated polysaccharides, the sulphate from the mucilage was lost during methylation and it was impossible to deduce the site of sulphate by methylation before and after desulphation.

\textbf{Methylation studies}

Two standard xylans (expt.12) containing 1,3-linked units and a mixture of 1,3- and 1,4-linked units respectively were methylated and
analysed to provide standards for the present studies. GLC analysis of the methylated mucilage $S^+$ (expt. 12) revealed the presence of the methylated sugars set out in tables 20 and 21.

Table 20  Methylated sugars from Rhodella mucilage ($S^+$)

<table>
<thead>
<tr>
<th>$T^a$ (OV-225)</th>
<th>Assignment as alditol acetates</th>
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</thead>
<tbody>
<tr>
<td>0.48</td>
<td>2,3,4-tri-O-methylrhamnose</td>
</tr>
<tr>
<td>1.02</td>
<td>2,4-di-O-methylxylose (major)</td>
</tr>
<tr>
<td>1.15</td>
<td>2,3-di-O-methylxylose (major)</td>
</tr>
<tr>
<td>1.70</td>
<td>3,4,6-tri-O-methylglucose</td>
</tr>
<tr>
<td>1.85</td>
<td>2,4,6-tri-O-methylgalactose</td>
</tr>
<tr>
<td>1.96</td>
<td>2,3,6-tri-O-methylgalactose</td>
</tr>
<tr>
<td>2.10</td>
<td>2,3,6-tri-O-methylglucose</td>
</tr>
<tr>
<td>2.90</td>
<td>2,6-di-O-methylhexose</td>
</tr>
<tr>
<td>3.30</td>
<td>3,6-di-O-methylhexose</td>
</tr>
</tbody>
</table>

(a) $T =$ retention time relative to 1,5-di-O-acetyl-2,3,4,6-tetra-O- methylglucitol.

Table 21  Methylationed sugars from Rhodella mucilage ($S^+$)

<table>
<thead>
<tr>
<th>$T^b$ (15% BDS)</th>
<th>Assignment as methylglycosides</th>
<th>$T^b$ (10% PPE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.74</td>
<td>2,3,4-tri-O-methylrhamnose</td>
<td>0.47</td>
</tr>
<tr>
<td>1.48; 1.92</td>
<td>2,4-di-O-methylxylose</td>
<td>0.75; 0.9</td>
</tr>
<tr>
<td>1.58; 1.78</td>
<td>2,3-di-O-methylxylose</td>
<td>0.66; 0.75</td>
</tr>
<tr>
<td>2.9 ; 4.3</td>
<td>3,4,6-tri-O-methylglucose</td>
<td>1.7</td>
</tr>
<tr>
<td>4.0 ; 4.6</td>
<td>2,4,6-tri-O-methylgalactose</td>
<td>2.0 ; 2.4</td>
</tr>
<tr>
<td>3.2 ; 4.3</td>
<td>2,3,6-tri-O-methylgalactose</td>
<td>1.7 ; 2.2</td>
</tr>
<tr>
<td></td>
<td>2,6-di-O-methylhexose</td>
<td>3.8</td>
</tr>
</tbody>
</table>

(b) $T =$ retention time relative to 2,3,4,6-tetra-O-methyl- $\beta$-methylglucoside.
These results show that the rhamnose is present solely as end group, that the xylose occurs as 1,4- and 1,3-linked units, some of the 1,4-linked units carrying methoxyl at C-3. The galactose is both 1,3- and 1,4-linked and the glucose is both 1,2- and 1,4-linked. Both the galactose and the glucose may occur at branch points 1,3,4- and 1,2,4-linked.

Reduction of the partially methylated polysaccharide with lithium aluminium hydride [expt. 12 (b) i,ii] caused extensive degradation and failed to produce any new peaks as glycosides or alditol acetates on GLC analyses (see conclusion reached on S19 polysaccharide, p.90) although the peaks corresponding to methylated uronic acids were considerably reduced. This was also true for the reduction with boro-deuteride [expt. 12 (b) iii] of the methylated autoclaved materials, except that the peak with $T = 1.7$ (as alditol acetate) had increased. However, GC-MS analysis showed this now comprised a mixture of degradation products as well as the 3,4,6-tri-$O$-methylhexose.

Reduction of the mucilage via the carbodiimide complex and methylation of the resulting neutral polysaccharide, isolated in 72% yield, showed a 2-fold increase in the peak with $T = 1.7$ as alditol acetate compared with that of the initial polysaccharide. GC-MS showed this to be a mixture of 3,4,6-tri-$O$-methylhexose and 2,4,6-tri-$O$-methylglucose. In this case, no evidence for degradation products was observed in this peak. Thus from these results, it can be concluded that the glucuronic acid is 1,3-linked and possibly 1,2-linked as well, although 1,2-linked glucose had been identified in the initial polysaccharide.
Periodate oxidation

Periodate oxidation of the sulphated polysaccharide \( (S^+) \) gave a reduction of periodate of 0.5 mole for every anhydro sugar residue (see graph, p.140). Similarly, the mucilage \( (S^-) \) which had been cultured in the absence of sulphate and was devoid of sulphate consumed 0.6 mole for every anhydro sugar residue. Due to lack of material, the reaction could be followed by only one method, that is, by UV absorptiometry.

The polyalcohols were recovered in good yield \( (S^+ \text{ polyol I } 63\%) \) \( (S^- \text{ polyol I } 72\%) \). These each gave, on hydrolysis, glucuronic acid, xylose, galactose, 3-O-methylxylose, glucose (trace), mannose (trace), rhamnose (trace) and glycerol. The proportion of galactose and xylose appeared from the paper chromatograms and GLC peaks to have decreased and the 3-O-methylxylose to have increased. The glycerol could be derived from 1,4-linked xylose and/or 1,2-linked glucose units.

The polyalcohols were subjected to a second periodate oxidation and it was found that they each reduced a further 0.2 mole of periodate per anhydro sugar unit indicating that a certain amount of acetal formation had hindered complete periodate oxidation.

The polyalcohols \( (S^+ \text{ polyol II, 7.5 mg}) \) \( (S^- \text{ polyol II, 6.3 mg}) \) were recovered in only about 30\% yield indicating that cleavage of polysaccharide chains had occurred with the production of small units which were lost on dialysis. This may be due to \( \beta \)-elimination of some of the uronic acid units during the alkaline conditions of reduction of the polyaldehyde.
Hydrolysates of polyalcohols II were similar to those of polyalcohols I except that rhamnose and mannose were absent from the hydrolysate of $S^-$ polyol II. These results are in general agreement with those from methylation studies. They confirm that a proportion of the xylose and galactose units are 1,3-linked, that the rhamnose is vulnerable to periodate oxidation, and therefore can be present solely as end-group.

Paper electrophoresis of the $S^+$ and $S^-$ polyols II hydrolysates showed the presence of glucuronic acid and aldobiuronic acid but neither erythronic acid nor glyceric acid could be detected indicating that all the glucuronic acid, at least in these polyols, were 1,3-linked.

**Partial hydrolysis studies**

Two partial hydrolysates were carried out although the amount of mucilage available made any large scale experiments impossible. From hydrolysates of 20 mg mucilage (expt. 15a), a mixture of oligouronic acids were eluted from the base line of a 3 M paper chromatogram. The neutral sugars had been separated by elution of the sugar in a basic solvent. The mixture of oligouronic acids were esterified, reduced and hydrolysed. Paper and gas-liquid chromatographic analyses revealed that the major sugar was glucose, presumably derived from glucuronic acid. Xylose, galactose and 3-O-methylxylose were also present. The uronic acid must therefore be linked to both xylose and galactose confirming that the mucilage is indeed a heteropolysaccharide.

In a second partial hydrolysis (30 mg) (expt. 15 b), the hydrolysate was separated by preparative paper chromatography in an acid solvent. An oligouronic acid, $R_{gal} 0.5$, was eluted from the chromatogram.
Electrophoresis at pH 6.7 gave a single spot $M_{\text{Glc UA}}^{0.5}$. It had a DP of 2.0. After esterification, reduction, and hydrolysis paper and gas-liquid chromatographic analyses revealed the presence of glucose and galactose and a trace of xylose. This is further evidence of the glucuronosyl linkage to galactose in the mucilage.

**Conclusion and comparison with other red algal polysaccharides**

In conclusion, evidence has been presented that the extracellular mucilage produced by *Rhodella maculata* is a sulphated heteropolysaccharide containing mainly xylose and glucuronic acid together with smaller proportions of $3-O$-methylxylose, rhamnose, galactose and glucose. The xylose units are 1,4- and 1,3-linked, some of the 1,4-linked units carrying methoxyl at C-3. The glucuronic acid occurs as 1,3-linked units. Rhamnose is present as end-group together with 1,3- and 1,4-linked galactose; and 1,2- and 1,4-linked glucose. Branch points are occupied by 1,3,4- and 1,2,4-linked galactose and/or glucose units. Although all attempts to fractionate the material into more than a single polysaccharide were unsuccessful, no evidence that glucose is linked to the other sugars or to the uronic acid has been advanced.

Glucuronic acid has been reported in a variety of red algal polysaccharides, but as far as the author is aware, no evidence for its linkage has been reported. Nunn and his colleagues reported the presence of glucuronic acid linked to C-4 of galactose in the polysaccharide from the red alga *Anatheca dentata*. Xylose has been reported as a minor component in a number of agars, and in the galactan from *Anatheca dentata* (Solieriaceae). In the latter work the xylose units are either 1,4-1,2-linked and/or present as non-reducing end groups. Xylose also occurs in galactans from the Grateloupiaceae species. Its presence as the sole component of red algal xylans has already been pointed out (see p.114), but this is the first time a sulphated polysaccharide from this or any other group of algae has been shown to contain xylose and glucuronic acid as the main constituents with a smaller proportion of rhamnose, $3-O$-methylxylose, galactose and glucose.

The author is grateful to Mr. I. Tibbs of the Biochemistry department for carrying out the amino acid analysis.
Appendix One

Standard graphs.  Pages 144-152
Absorbance at 1.44 nm

Phenol-sulphuric acid standard graphs
Unicam: SP500.

Xylose

Glucose

→ µg/ml
Pyruvic acid standard graph

LDH method

Unicam SP 500

$\text{mg}$ of pyruvic acid.
Difference in absorbance at 0.3 25mm.

Sulphate determination
Standard graph by
Jones and Letham method.

Uninam 58500

μg of sulphate.
meta-hydroxy diphenyl method
SP 500

Absorbance at 520nm

Glucurone

Glucoronic acid

Hydrol
Absorbance at 530nm

Carbazole standard graphs
Unicam SP500

Glucone

Gluconic acid

\[ \mu g/mL \]
Appendix Two: Paper chromatographic mobilities relative to glucose. (see p.44 for details of solvent system).

<table>
<thead>
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<th></th>
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<td>Galacturonic acid</td>
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<td>2-0-methylxylose</td>
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<td>3-0-methylfucose</td>
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<td></td>
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<td>1.3</td>
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</table>
Appendix Two: Retention times of trimethylsilyl derivatives relative to xylitol-TMS derivative on Apiezon K.

<table>
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<th>Trimethylsilyl derivatives of</th>
<th>Retention Time T</th>
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<td>xylitol</td>
<td>1.00</td>
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<td>glucitol</td>
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<tr>
<td>galactitol</td>
<td>2.0</td>
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<tr>
<td>mannitol</td>
<td>1.9</td>
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<td>rhamnitol</td>
<td>1.3</td>
</tr>
<tr>
<td>3-O-methylxylitol</td>
<td>1.0</td>
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<tr>
<td>arabinitol</td>
<td>1.2</td>
</tr>
<tr>
<td>xylose</td>
<td>1.5; 2.0</td>
</tr>
<tr>
<td>glucose</td>
<td>2.7; 4.5</td>
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<tr>
<td>galactose</td>
<td>2.0; 2.7</td>
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<tr>
<td>mannose</td>
<td>1.6</td>
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<tr>
<td>rhamnose</td>
<td>0.9; 1.3</td>
</tr>
<tr>
<td>3-O-methylxylose</td>
<td>0.9; 1.1</td>
</tr>
<tr>
<td>fructose</td>
<td>1.7; 2.3</td>
</tr>
<tr>
<td>6-O-methylgalactose</td>
<td>1.8</td>
</tr>
<tr>
<td>arabinose</td>
<td>0.8; 0.9</td>
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<tr>
<td>4-O-methylglucose</td>
<td>2.0; 3.4</td>
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<td>apiose</td>
<td>0.7; 1.1</td>
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<tr>
<td>ribose</td>
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</table>
Appendix Three  Polyacrylamide gel electrophoresis

The Shandon SAE-2734 analytical polyacrylamide electrophoresis apparatus was used in this experiment. Shandon general instructions were followed:-

Solution 1. Running buffer for small pore gel.

This contains
a. hydrochloric acid (1N, 48 ml).
b. 2-amino-2-hydroxymethyl-1,3-propanediol (TRIS) added to give pH 8.9.
c. N,N,N',N'-tetramethylethylenediamine (TEMED) (0.46 ml).
d. water (made up to 100 ml).

Solution 2. Stacking buffer for large pore gel.

a. hydrochloric acid (1N, 48 ml).
b. TRIS added to give pH 7.0.
c. TEMED (0.46 ml).
d. water (made up to 100 ml).

Solution 3. Electrode buffer.

a. TRIS (6.0 g).
b. glycine (28.8 g).
c. water to 1 litre.

This solution (pH 8.3) was diluted 1:10 before use. Usually about 550 ml was required per run.

Solution 4. Small pore gel acrylamide solution.

a. acrylamide (30.0 g) (POISON).
b. N,N'-methylene bis acrylamide (BIS) (0.735 g).
c. water to 100 ml.
Solution 2. Large pore gel acrylamide solution.
   a. acrylamide (10.0 g).
   b. BIS (2.5 g).
   c. water to 100 ml.

Solution 6. Ammonium persulphate initiator.
   a. ammonium persulphate (0.14 g).
   b. water to 100 ml.

Solution 7. Riboflavin initiator.
   a. riboflavin (4 mg).
   b. water to 100 ml.

Solution 8. Sucrose solution.
   a. sucrose 40 g.
   b. water to 100 ml.

Solution 2*. Bromophenol blue marker.
   a. bromophenol blue (1 mg).
   b. water to 100 ml.

One drop of this solution was added to the upper buffer reservoir.

The above solutions were all kept at 2°C until used. Working
solutions were made as follows:— The small pore gel (7.5% acrylamide
running gel) consists of
   a. solution 1. (6 ml).
   b. solution 4. (6 ml).
   c. solution 6. (12 ml).
The large pore gel (2.5% acrylamide stacking gel) consists of
a. solution 2 (3 ml).
b. solution 5 (6 ml).
c. solution 7 (3 ml).
d. solution 8 (12 ml).

Staining methods
1. 0.1% wt/vol toluidine blue. Gels were soaked in this staining solution for 2 - 6 hrs, then washed repeatedly with water.

2. 1% wt/vol toluidine blue. Gels were soaked in this solution for 15 mins, then washed repeatedly with water.

3. Stains-all: The stock solution was a 0.1% wt/vol of "stains-all" (Serva) in formamide. A working stain solution was made by diluting 10 ml of the stock solution with 90 ml of 100% formamide followed by 100 ml of water. These solutions must be protected from light. Gels were stained overnight in the dark and then destained in running tap water out of direct light.

4. Coomassie blue
Solution 1. trichloroacetic acid (12.5 g) in water (100 ml).
Solution 2. coomassie blue (0.5 g) in water (50 ml).
Solution 3. solution 2 diluted 1:20 with solution 1.
The gels were fixed in solution 1 for 30 mins. They were then stained in solution 2 for 2 - 6 hrs. Destaining was achieved by washing with solution 1.
2. Periodate-Schiff reagent

Basic Fuchsin solution:

Solution 1: 0.1% Fuchsin saturated with SO₂.

Solution 2: 0.05M mercuric chloride.

Solution 3: 0.1N sulphuric acid.

Basic Fuchsin solution was made by mixing solution 1 (1 ml), solution 2 (1 ml) and solution 3 (10 ml) and adding water to 100 ml.

Solution 4: 12.5% trichloroacetic acid in water.

Solution 5: 1% periodic acid in 3% acetic acid.

Solution 6: 0.5% metabisulphite solution.

The gels were immersed in solution 4 for 30 mins at room temperature, then rinsed with water, and immersed in solution 5 for 50 mins. They were then washed until free of iodate (UV absorption). The gels were stained in basic Fuchsin-sulphite solution for 50 mins in the dark, washed with solution 6 several times and stored in 3% acetic acid.
Appendix 4. Attempted enzymic hydrolysis of alginate

Introduction

Alginic acid is a polyuronide consisting of D-mannuronic acid and L-guluronic acid. Although its structure is not completely established, the polymer has been shown to contain mannuronic acid blocks, β-1,4-D-mannuronosyl-D-mannuronate, guluronic acid blocks and alternating blocks, that is, 1,4-D-mannuronosyl-L-guluronate. The structure of the polymer is not completely understood, but it is known to contain mannuronic acid blocks, guluronic acid blocks, and alternating blocks.

Alginic acid is known to be a difficultly hydrolysable substance; acid hydrolysis, for example, is accompanied by extensive decomposition.

Enzymes hydrolysing alginic acid have been studied since 1931. Waksman and Allen isolated alginic acid decomposing bacteria from soil and sea-water. These bacteria were claimed to hydrolyse alginic acid into smaller groups of mannuronic but not into single units.

Thereafter only a few reports appeared merely noting the presence of alginase. However in 1954, Kooiman isolated a bacterium capable of hydrolysing alginic acid to "mannuronic acid and oligo-uronides," shown by paper chromatography only. Yashikawa obtained two kinds of enzymes both capable of hydrolysing alginic acid as shown by the decrease in viscosity and increase in reducing power of the digests. Similar criteria for evidence of alginase activity have also been given by several other workers. The alginase isolated by Yashikawa, however, produced comparatively less reducing power and was considered to be an endoenzyme.

From paper chromatographic evidence, mannuronic acid was thought to be the end-product from alginic acid after digestion with the alginase from Agarbacterium alginicum. However alginsates from other
microorganisms were found to produce no mannuronic acid but a series of oligosaccharides containing an unsaturated uronic acid on the non-reducing end of the oligosaccharide chain. Similar action was observed for the abalone alginase.

In recent years, a greater understanding of alginase activity has been obtained. Nakada and Sweeney separated two alginases, I and II, from abalone hepatopancreas. Alginase I appeared to be specific for \( \beta-1,4 \)-links involving D-mannuronic acid, and alginase II for \( 1,4 \)-bonds involving L-guluronic acid. Alginases I and II could not degrade alginic acid completely indicating other types of bonds. As with the other alginases, alginases I and II seem to require a high ionic concentration. This seems to be the only common requisite for alginase activity. Their optimum pH temperature and heat susceptibilities vary greatly.

The action of alginases is further complicated by the presence of alginic acid epimerases and the difference in fine structures of alginic acids from different algae.

Thus, no alginase hydrolysing alginic acid to its constituent monomers has been found. In conclusion, alginases so far studied are more properly called eliminases, depolymerases or epimerases.

The mixture of enzymes from Sclerotium rolfsii has proved useful in hydrolysing to the monomers various uronic acid containing polysaccharides which had previously been partly acid hydrolysed. In an attempt to hydrolyse sodium alginate to its constituent monomers in quantitative yield, digests of the partly acid hydrolysed polymer and \( S. \) rolfsii enzymes were prepared. It was aimed to achieve complete hydrolysis of alginic acid so that the proportion of mannuronic acid to guluronic acid could be found rapidly and accurately.
Experimental

Expt. 1. Formic acid and enzymes hydrolysates.

Sodium alginate (500 mg) was hydrolysed with 90% formic acid (25 ml) at 100° for 6 h [CM 3a]. The hydrolysate was dialysed extensively to give a mixture of soluble and insoluble alginic acid in the dialysis sac. This was neutralised with 1M-sodium hydroxide to give a clear solution. The carbohydrate content of this solution was determined by the phenol-sulphuric acid test [CM 2a] (recovery = 80%).

The partially hydrolysed sodium alginate free from any monouronic acid (12 mg aliquots was added to the 10 mM sodium acetate buffers (pH 3.64, 4.06, 4.25, 4.48, 4.85 and 5.61) containing 100 mg of dialysed and freeze-dried enzyme mixture. Each of the enzyme digests was incubated at 30° for 24 h. The reaction mixtures were treated with Amberlite resin IR 120 (H⁺) to remove Na⁺ ions and then evaporated to dryness. The syrups were examined by paper chromatography [CM 5(a) i].

Expt. 2. Sulphuric acid and enzymic hydrolysates.

Sodium alginate (0.8 g) was hydrolysed with 0.5M-sulphuric acid at 100° for 4 h. The mixture was diluted with water, and after centrifugation, the centrifugate was rehydrolysed. This procedure was repeated twice more and the total hydrolysate was neutralised with barium carbonate. The neutralisate was treated with Amberlite resin IR 120 (H⁺) to give a mixture of soluble and insoluble alginic acid.

The soluble alginic acid separated by centrifugation was examined by paper chromatography and subjected to enzymic hydrolysis at pH 4.5 without previous dialysis as in Expt. 1 above.
Expt. 3. Trifluoroacetic acid and enzymic hydrolysis

Sodium alginate (0.8 g) was hydrolysed with 1M-trifluoroacetic acid (5 ml) for 1.5 h at 100°. After hydrolysis, the trifluoroacetic acid was removed by evaporation of the solution to dryness. The sample was kept in a vacuum desiccator overnight in the presence of KOH pellets to remove any traces of trifluoroacetic acid. The derived partial hydrolysate was subjected to enzymic hydrolysis at pH 4.5. as in Expt. 1.

Results and Discussion

The partially hydrolysed sodium alginate (expt. 1) shown to be free from monouronic acid by paper chromatography gave spots corresponding to mannuronic and guluronic acids after enzymic digestion with the Sclerotium rolfsii enzyme mixture. The intensities of these spots were stronger when the digests were carried at pH 3.64 - 4.48 than when they were carried at pH 4.85 - 5.61. Base-line material on the chromatograms showed incomplete hydrolysis. No spots were observed between the base-line material and the monouronic acids, indicating the absence of unsaturated oligouronides.

Enzymic hydrolysis of the sulphuric acid partial hydrolysate followed by paper chromatography showed that the spots due to monouronic acids had increased during enzymic treatment again complete hydrolysis was not achieved.

The trifluoroacetic acid hydrolysate did not dissolve in the enzyme digest and paper chromatography showed very little monouronic acid after incubation.
These results show that *Sclerotium rolfsii* enzymes hydrolyses sodium alginate. However the extent of hydrolysis is too small to allow a quantitative determination for the molar ratio of mannanuronic and guluronic acids in alginic acid.

The author is indebted to Dr. F. Bateman of Cornell University for the culture of *Sclerotium rolfsii*. 
1. B.A.D. Stocker in Bacterial Anatomy (1956) 19.


   Ed. I.C. Gunsalus and R.Y. Stainer, Acad. Press, N.Y.

   The Williams and Williams Co., Baltimore.


   Adv. in Chemistry series No. 117.

    Ed. W. Pigman and D. Horton.

21. L.F. Leloir, C.C. Cardini and E. Cabib, Comparative Biochemistry,


    233 (1958) 783.

24. E.E.B. Smith, G.T. Mills, H.P. Bernheimer, and R. Austrian,

    (US), 45 (1959) 905.

    Camb., Mass.


    Ed. W. Pigman and D. Horton, Acad. Press.


38. W.C. Boyd (1943), Fundamentals of Immunology, Interscience, Inc. N.Y.


70. O. Luderitz, K. Jann, R. Wheat, Comprehensive Biochemistry, 26A (1968) 105.


89. H.G. Hedrick, Phytopath. 46 (1956) 14-5.


Sugar Cane disease of the world; Ed. J.P. Martin et al., Elsevier, N.Y.
McGraw-Hill Inc.
and D. Parkinson, Toronto Univ., Toronto Press.


127. CA: 70: 943e.


135. K.A. Sabet and W.J. Dowson, Nature 168 (1951) 605.


140. CA: 64: P: 19976C.


205. B. Lindberg, J. Lonngren and W. Nimmich, Carb. Res. 23 (1972) 47.


220. H. Kylin, _Z. physiol. chem._ 83 (1913) 171.


252. R.M. Zacharius, T.E. Zell, J.H. Morison, J.J. Woodlock, 


STUDIES ON THE SYNTHESIS AND COMPOSITION OF EXTRACELLULAR MUCILAGE IN THE UNICELLULAR RED ALGA RHODELLA

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SUMMARY

$^{35}$S$\text{O}_3^{2-}$ has been used to investigate the production of extracellular mucilage by log-phase cells. Uptake of isotope occurs most rapidly in the light, when cells are actively dividing. The mucilage comprises about 50% carbohydrate, 16% protein and 10% sulphate. The major sugar is xylose; uronic acid, a small amount of galactose, glucose (trace) and 2 reducing substances are also present. Methylation studies have established the major linkages. Electron-microscope autoradiography shows that the mucilage is packaged in the Golgi bodies, passing to the plasmalemma in large vesicles. Sulphation of the mucilage occurs in the Golgi cisternae.

INTRODUCTION

The characteristic polysaccharides of red algae are by now well known to be galactans consisting of alternating 1,3- and 1,4-linked galactose residues carrying variable proportions of half ester sulphate and methyl ether residues (Percival & McDowell, 1967). Xylans, comprising 1,3- and 1,4-linked xylose units have also been found in a number of species (Turvey & Williams, 1970). Some studies on the derivation and localization of red algal polysaccharides have been carried out (e.g. Peyrière, 1969, 1970; McBride & Cole, 1971; Chamberlain & Evans, 1973; and Gordon & McCandless, 1973). However, with the exception of the work of Loewus, Wagner, Schiff & Weistrop (1971) on Chondrus and of Ramus (1972) and Ramus & Groves (1972) on Porphyridium, little experimental work has been reported on the synthesis of red algal polysaccharides within the cells, rates of production and subsequent sequential movement of these to the outside under different conditions, nor are there detailed chemical analyses of the secreted products. The present communication is a report of an investigation carried out on the synthesis and composition of the mucilage encapsulating the red algal unicell Rhodella maculata.
MATERIALS AND METHODS

Isolate

Cultures have been maintained in the laboratory in Leeds from the original made from a sample of sand, as described by Evans (1970).

Culture conditions

The cells were grown in either SWM3 medium (Provasoli, McLaughlin & Droop, 1957) or in ASP12 medium (Provasoli, 1964). In experiments where the amount of sulphate supplied was varied ASP12 was used, MgSO₄ being replaced by MgCl₂. Cultures of 100 ml in 250-ml flasks were kept in suspension by gentle shaking and illuminated by 4000 lx from Northlight fluorescent tubes with a 16-h photoperiod.

Light microscopy

Cells were fixed in 10% acrolein and embedded in glycol methacrylate, as described by Evans & Holligan (1972). One-micron sections were stained as follows: 0.05% toluidine blue at pH 6.8 (O'Brien, Feder & McCully, 1964), periodic acid/Schiff (PAS) (Feder & O'Brien, 1968), alcian blue and alcian yellow (Parker & Diboll, 1966; Scott & Dorling, 1965). The same histochemical tests were also performed on whole cells which had been fixed in 4% formaldehyde for 30-90 min.

Electron microscopy

Cells were fixed in 5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.0) containing 0.25 M sucrose, post-osmicated, dehydrated and embedded in Epon 812 as previously described (Evans, 1970). Sections were cut out with a diamond knife on an LKB Ultratome, stained with Reynolds lead citrate and examined with a Siemens Elmiskop IA electron microscope.

Isolation of mucilage

Cultures were centrifuged at 10000 g for 15 min to pellet the cells. The supernatant was passed through 0.2-μm Millipore filters, and dialysed for 48 h against running tap water and 24 h (2 changes) against deionized water. The polysaccharide (solubilized mucilage) was re-filtered through 0.45-μm Millipore filters and freeze-dried to a white solid (yield 200 mg 1. of culture medium after 20 days) or precipitated with 4 vol. of absolute ethanol and recovered by centrifugation at 40000 g for 30 min. Mucilage associated with the pellet of cells (encapsulating mucilage) was removed by shaking with water at 40 °C for 48 h. After centrifugation at 50000 g for 30 min the supernatant was filtered, dialysed and recovered as above.

Labelling with ³⁵SO₄²⁻

Cells grown in ASP12 medium were centrifuged at 1000 g for 10 min, resuspended in ASP12 containing 10⁻⁴ M SO₄²⁻, centrifuged and again resuspended in the latter medium to which carrier-free ³⁵SO₄²⁻ was added at 1 μCi/ml. There were approximately 3 x 10⁶ cells per ml culture. At the various time intervals 10-ml samples were removed and the cells pelleted by centrifugation at 10000 g for 15 min. Mucilage solubilized in the medium was recovered as described above. The final supernatant was counted to give an estimate of the amount of sulphate uptake, measured as removal from the medium. The pelleted cells were washed 3 times in medium containing 1 M SO₄²⁻ in order to displace any adsorbed isotope and then resuspended in 80% ethanol. After resuspending twice more in 80% ethanol the supernatants were pooled (EtOH-insol. fraction). The EtOH-insol. fraction was hydrolysed in 1 N sulphuric acid for 36 h in sealed hydrolysis vials and neutralized with BaCO₃ prior to counting.
Mucilage production in Rhodella

Polysaccharide estimation

The phenol-sulphuric method of Dubois et al. (1956) was used. Standard solutions contained equal amounts of glucuronic acid and xylose.

Polysaccharide counting

0.1-ml samples were placed on 2.1-cm GF/A disks and counted in 5 ml 0.4% PPO plus 0.01% POPOP in toluene in a Nuclear Chicago Unilux II scintillation spectrometer.

Autoradiography

Cells grown in ASP12 were transferred to ASP12 minus sulphate overnight, then centrifuged and resuspended in ASP12 containing $10^{-1} \times SO_4^{2-}$ and 50 $\mu$Ci/ml carrier-free $SO_4^{2-}$ ($3 \times 10^9$ cells/ml). For light-microscope autoradiography cells were fixed and embedded as described above and autoradiographs prepared as described previously (Evans, Simpson & Callow, 1973). For EM autoradiography the method described by Callow & Evans (1974) was followed.

Analytical methods

The general analytical techniques were as described in a previous publication (Cleare & Percival, 1972). In addition, for paper chromatography the following solvent systems were used, v/v: (K) butan-1-ol:acetic acid:water (4:1:5) to separate xylose and 6-O-methylgalactose; (L) ethyl methyl ketone saturated with water and containing 3% cetylpyridinium chloride to distinguish sulphated fragments (Rees, 1959), and sprayed with (7) urea-hydrochloride (Bell, 1955) for ketoses. The sulphate content was determined on formic acid hydrolysates, and on the ash, with 4-amino-4'-chlorodiphenylamine HCl (K and K Laboratories, Hollywood, California) (Jones & Letham, 1954). Uronic acid was assayed by the modified carbazole method (Bitter & Muir, 1962). Specific rotations were measured in H_2O at 20 °C in a Perkin-Elmer 141 polarimeter using the sodium D-line. The carbohydrate content was determined by the phenol-sulphuric acid method (Dubois et al. 1956) and the absorption read off a standard xylose (4 parts), glucuronic acid (1 part) graph. Unless otherwise stated, hydrolysates were with 90% formic acid (Cleare & Percival, 1972). Glucose and galactose were assayed with glucose oxidase (Salton, 1960) and galactosstat (Worthington enzyme reagents), respectively. Demethylation was by the method of Bonner, Bourne & McNally (1960).

Analysis of the mucilage

Samples (5 mg each) of the different batches were hydrolysed and the hydrolysates were analysed by paper chromatography (solvents A-D; sprays 1-3 and 6), by ionophoresis, and by gas-liquid chromatography (g.l.c.) of the derived TMS derivatives. The sugars in the hydrolysates were reduced to the corresponding alditols and the latter analysed by g.l.c. as the TMS derivatives. An aliquot of a hydrolysate was esterified, reduced and rehydrolysed, and the derived sugars analysed by chromatography (spray 1 and 3) and the glucose assayed with glucose oxidase.

The mucilage (19.8 mg) was hydrolysed and the derived sugars and oligosaccharides were separated by preparative paper chromatography (solvent C). Each fraction was examined by paper chromatography, and an aliquot was converted into the TMS derivative and analysed on g.l.c. (column 3). The mixture of oligosaccharides was esterified, reduced and hydrolysed. The hydrolysate was analysed as for the individual sugars.

To confirm the presence of 3,6-anhydrogalactosidic linkages the polysaccharide (9.8 mg) was treated with 1 N H_2SO_4 at 70 °C for 2 h. Ethanol (6 vol.) was added to the cooled solution and the resulting precipitate removed by centrifugation and hydrolysed. The supernatant solution was neutralized (BaCO_3) and the derived filtrate examined by paper chromatography. It was rehydrolysed with 1 N H_2SO_4 at 100 °C for 4 h and the hydrolysate worked up as before and analysed by paper chromatography.

1-2
Fractionation experiments

Chromatography. The mucilage (1.2 mg) in water (2 ml) as a homogeneous gel was applied to a column (0.9 x 16 cm) of DE52 cellulose (12 g) and the column eluted with water, followed by elutions with 0.3 M KCl. Fractions were monitored for carbohydrate with phenol-sulphuric acid (Dubois et al. 1956).

Ultracentrifugation. The mucilage (0.3% solution in 0.1 M NaCl) was analysed on a Beckman Spinco Model E ultracentrifuge at 20000 and 33000 rev/min.

Assay of 3,6-anhydrogalactose

Aliquots (2 ml) of the mucilage, of the desulphated mucilage [5 mg each in water (20 ml)], and of xylose and glucuronic acid (2.5 and 0.75 mg, respectively) in water (20 ml), were treated separately with resorcinol reagent (10 ml) according to the method of Yaphe (1960) and the absorptions read at 550 nm.

Determination of ash content and the sulphate content of the ash

The mucilage (18 mg) was converted into the free acid form by agitation of an aqueous solution with Amberlite IR 120 (H+) resin. The resulting solution was neutralized to pH 7 with sodium hydroxide solution and then freeze-dried (17 mg). It was incinerated in a platinum crucible to constant weight. The ash was dissolved in a known volume of water and the solution assayed for sulphate.

Desulphation of the mucilage

The polysaccharide (31.6 mg) in water (20 ml) was treated with potassium borohydride (19 mg) with shaking for 20 h at room temperature. Additional borohydride (30 mg) and sodium hydroxide (10 ml, 3 N) was added and the mixture heated at 80 °C for 8 h (Rees, 1961). The cooled mixture was set aside overnight at room temperature and the solution dialysed. The material (24 mg) remaining in the dialysis sac was recovered by freeze-drying, and analysed for sulphate. The dialysates (1800 ml) were concentrated, after neutralization, to 12 ml and the carbohydrate content (2.5 mg) determined. Cations were removed with Amberlite IR-120 (H+) resin and the resulting solution was assayed for SO4^{2-}.

Methylation of the mucilage

The mucilage (16.3 mg) was methylated by the modified Hakomori procedure (Bjorndal & Lindberg, 1969). The product was hydrolysed and an aliquot of the hydrolysate was converted into the methyl glycosides and analysed by g.l.c. (columns 1 and 2). The remainder of the methylated hydrolysate was reduced and converted into the methylated alditol acetates (Bjorndal & Lindberg, 1969) which were analysed by g.l.c. (column 4) and by g.l.c./mass spectrometry (Cleare & Percival, 1972) (column 7).

Methylated polysaccharide prepared as before was reduced with lithium aluminium hydride (Mian & Percival, 1973) and then remethylated. The product was hydrolysed and the hydrolysate analysed as for the unreduced methylated hydrolysate.

RESULTS

The general structure of Rhodella has been described previously (Evans, 1970). Characteristic features are a large central pyrenoid to which lobes of the single chloroplast are attached by isthmuses, and an eccentrically placed nucleus (Fig. 6). Outside the plasmalemma there is a thick mucilaginous envelope (Fig. 7). The peripherally situated Golgi bodies are characteristically associated (at the forming face) with an arc of endoplasmic reticulum (ER) which is continuous with that in the
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cytoplasm and with the subplasmalemmal anastomosing network of ER (Fig. 8). Projections from the latter approach the plasmalemma and fuse with it. Electron-dense material may sometimes be seen in the mucilage, where the ER projections open through the plasmalemma. The ER associated with the forming face of the Golgi bodies appears to produce many small vesicles with electron-dense content. These lie in close proximity to the cisternae at the forming and lateral faces of the Golgi body (Fig. 9). At the maturing face the cisternae become more distended and contain finely fibrillar material. The cisternae cut off from the maturing face round off to give membrane-bounded vesicles, the content of which is fingerprint-like in appearance and closely resembles the encapsulating mucilage. Such vesicles are seen closely pressed to the Golgi surface (Fig. 9) and in the region of cytoplasm between the Golgi bodies and the plasmalemma (Fig. 10). The vesicles appear to discharge their contents by exocytosis, thereby supplementing the external mucilage envelope.

Histochemistry of the encapsulating mucilage

The results are summarized in Table 1. $\gamma$-Metachromasia with toluidine blue indicates acidic macromolecules with well defined negative charges. The most commonly reacting groups are sulphate, phosphate and carboxyl, in descending order. The positive reaction with alcian blue indicates that the negative charge is primarily due to sulphate. The very intense reaction with alcian yellow indicates that the material is strongly carboxylated. The weak reaction with PAS indicates the presence of polysaccharide with some free hydroxyl groups on vicinal carbon atoms. All the protein stains used gave negative results.

<table>
<thead>
<tr>
<th>Stain</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>Toluidine blue</td>
<td>$++$ ($\gamma$-metachromasy)</td>
</tr>
<tr>
<td>Alcian blue</td>
<td>$+$</td>
</tr>
<tr>
<td>PAS</td>
<td>$+$</td>
</tr>
<tr>
<td>Alcian yellow</td>
<td>$+++$</td>
</tr>
<tr>
<td>Fast green FCF</td>
<td>$-$</td>
</tr>
</tbody>
</table>

Reactions are graded: $-$, nil; $+$, weak; $++$, moderate; $+++$, strong.

Mucilage secretion

Cells subcultured from stationary phase cultures exhibit typical sigmoid growth, the log phase beginning after 2 days (Fig. 1). Aeration enhances the rate of cell division. There is no division in cultures maintained in the dark. The total amount of polysaccharide (encapsulating and solubilized) follows a similar pattern (Fig. 2), although it is seen from Fig. 3 that during log phase there is a decrease in the amount of polysaccharide synthesized when the results are expressed on a per cell basis. After division has ceased there is an increase in cell size (mean diameter 18 $\mu$m compared with 10 $\mu$m in log phase cells) and secretion of mucilage continues. The encapsulating
Fig. 1. Growth curves obtained after subculturing stationary phase cells, when incubated in dark, $\triangle$–$\triangle$; light, $\square$–$\square$; and light with aeration, $\bigcirc$–$\bigcirc$.

Fig. 2. Polysaccharide secretion, as $\mu$g CHO/ml (glucuronic acid/xylose equivalents), $\bullet$–$\bullet$, after subculturing stationary phase cells; $\bigcirc$–$\bigcirc$, cell numbers.
mucilage (Fig. 7) has a mean thickness of 12 μm in stationary phase cells. Mucilage is not synthesized by cells in the dark, and although the encapsulating layer decreases to a mean thickness of 5 μm after 10 days, the cells never become naked.

![Graph](image1)

**Fig. 3.** Polysaccharide secretion, as μg CHO/cell, after subculturing.

![Graph](image2)

**Fig. 4.** Uptake of $^{35}$SO$_4^{2-}$ from ASP medium containing $10^{-4}$ M SO$_4^{2-}$; in the dark, □; and light, ○.

![Graph](image3)

**Fig. 5.** Distribution of radioactivity in cells incubated in $^{35}$SO$_4^{2-}$ in the light: ○, EtOH-sol.; ▲, EtOH-insol.; ○, secreted polysaccharide; in the dark: □, secreted polysaccharide.

Mucilage synthesis was investigated in log phase cells using inorganic sulphate $^{35}$SO$_4^{2-}$ in ASP12 medium to which $10^{-4}$ M carrier sulphate was added. In seawater there is negligible uptake, since the SO$_4$ concentration (0.28 mM) represents a 280-fold dilution of radioactivity, when $10^{-4}$ M carrier SO$_4$ is used. Uptake of $^{35}$SO$_4^{2-}$ measured
as removal from the medium, like cell division, is light-dependent (Fig. 4). Controls using dead cells did not take up radioactivity. Incorporation of $^{35}$SO$_4^{2-}$ into both the ethanol-soluble and ethanol-insoluble (includes encapsulating mucilage) fractions reached a constant level after 8 h of uptake (Fig. 5). However, incorporation into polysaccharides solubilized into the medium continued to increase in the light beyond this time, being 100 times greater than occurred in the dark. Uptake of sulphate thus proceeds most rapidly in the light when the cells are photosynthesizing and actively dividing, and exogenous sulphate is taken up, incorporated into polysaccharide and solubilized into the medium within a period of 2 h.

Table 2. Distribution of silver grains on autoradiographs following incubation in $^{35}$SO$_4^{2-}$

<table>
<thead>
<tr>
<th>Organelle</th>
<th>30-min pulse</th>
<th>30-min pulse/90-min chase</th>
<th>30-min pulse/180-min chase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% total grains</td>
<td>Relative activity</td>
<td>% total grains</td>
</tr>
<tr>
<td>Cytoplasm</td>
<td>8.0</td>
<td>1.0</td>
<td>8.0</td>
</tr>
<tr>
<td>Chloroplast</td>
<td>22.0</td>
<td>0.7</td>
<td>23.0</td>
</tr>
<tr>
<td>Pyrenoid</td>
<td>2.7</td>
<td>0.6</td>
<td>4.6</td>
</tr>
<tr>
<td>Starch grains</td>
<td>6.8</td>
<td>0.5</td>
<td>5.0</td>
</tr>
<tr>
<td>Nucleus</td>
<td>3.4</td>
<td>1.3</td>
<td>3.4</td>
</tr>
<tr>
<td>Vacuole</td>
<td>15.0</td>
<td>0.6</td>
<td>11.0</td>
</tr>
<tr>
<td>Mitochondria</td>
<td>1.0</td>
<td>1.6</td>
<td>0.5</td>
</tr>
<tr>
<td>Golgi</td>
<td>21.0</td>
<td>1.7</td>
<td>11.0</td>
</tr>
<tr>
<td>Vesicles</td>
<td>3.4</td>
<td>1.0</td>
<td>9.0</td>
</tr>
<tr>
<td>Miscellaneous</td>
<td>5.5</td>
<td>2.0</td>
<td>6.0</td>
</tr>
<tr>
<td>Mucilage A</td>
<td>10.0</td>
<td>0.5</td>
<td>16.0</td>
</tr>
<tr>
<td>Mucilage B</td>
<td>1.0</td>
<td>0.1</td>
<td>1.0</td>
</tr>
<tr>
<td>Mucilage C</td>
<td>0.5</td>
<td>0.1</td>
<td>2.0</td>
</tr>
<tr>
<td>Total no. grains</td>
<td>147</td>
<td>237</td>
<td>162</td>
</tr>
</tbody>
</table>

In order to obtain more precise information on the sequence of events involved in mucilage synthesis, autoradiographic studies were carried out. In cells incubated in medium containing $^{35}$SO$_4^{2-}$ for 30 min the Golgi bodies have a far greater activity than any other organelle (Table 2, Fig. 11). Following a 90-min (Figs. 12, 13) period in unlabelled medium, the relative activity of the Golgi bodies declines, with a corresponding increase in the activity of the peripherally situated detached Golgi vesicles (Table 2). After a longer chase period (180 min) (Fig. 14) activity in the Golgi has declined still further and that in the vesicles has also decreased. However, the relative activity of the mucilage has increased during the chase periods, when compared with the sample incubated for 30 min. (The seemingly low relative activity of the mucilage in electron micrographs is due to dilution of silver grains by its large volume.) This trend is confirmed by light micrographs of cells chased for 5 h (Fig. 15) where the encapsulating mucilage is seen to be heavily labelled.
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Chemical analysis

The extracellular mucilage from *R. maculata* freeze-dries to a white fibrous solid and gives a highly viscous 0.3% solution in water. It has a low positive specific rotation and comprises 72% of carbohydrate [(as glucose) or about 50% when read off a xylose (4 parts), glucuronic acid (1 part) graph] and about 16% of protein (calculated from a 2.51% N₂ content). It contains 12% by weight of uronic acid and 10% sulphate and yields 15% ash as the sodium salt.

Attempted separation of a neutral polysaccharide from acidic material by fractionation on DE52-cellulose (Bourne, Percival & Smestad, 1972) failed to yield any carbohydrate in the aqueous eluate. A complete recovery of carbohydrate was obtained by elution with 0.3 M KCl.

Table 3. Constituents separated and characterized from a hydrolysate of Rhodella

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Solvent Aniline oxalate spray, colour of spot</th>
<th>TMS derivative, T column 5</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Rₓ, Solvent</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>2.2</td>
<td>Pink</td>
</tr>
<tr>
<td>2</td>
<td>1.8</td>
<td>Light brown</td>
</tr>
<tr>
<td><em>3</em></td>
<td>1.45</td>
<td>Pink</td>
</tr>
<tr>
<td>4†</td>
<td>1.0</td>
<td>Brown</td>
</tr>
</tbody>
</table>

* [α]₅₅° was + 13° (cf. d-xylose, + 18°) and was identical with xylose run as control on paper and g.l.c. chromatograms.
† Identical with galactose run as control.

The gelling properties of the material prevented boundary formation in the ultracentrifuge.

Four batches of dialysed culture solution which yielded respectively 60, 59, 36 and 57 mg of freeze-dried material were investigated. Paper chromatography, ionophoresis and g.l.c. analysis of hydrolysates revealed the essential similarity of the different batches. Each contained xylose (major), uronic acid, and smaller amounts of galactose and glucose (trace) and 2 reducing substances with relatively high chromatographic mobilities (see Table 3). Only trace quantities of monouronic acid could be detected on paper chromatograms of hydrolysates, most of the uronic acid being degraded during hydrolysis. Several oligosaccharides were present and ionophoresis showed these to be oligouronic acids. Paper chromatography of the hydrolysate in solvent (L) confirmed the absence of any sulphated sugars. This solvent increases the mobility of sugar sulphates by as much as 10-fold (Rees, 1939). Amino acids were also detected in the hydrolysates. If the culture medium was not filtered through the 0.45/100 Millipore, fructose was also present in the hydrolysates.

Quantitative assay indicated less than 1% of glucose and about 3% of galactose in the hydrolysates, calculated on the weight of mucilage hydrolysed.

Each of the sugars was separated from a hydrolysate (see Table 3), and the identity of the xylose as d-xylose confirmed by its specific rotation, and by its paper chromatograms.
graphic and g.l.c. mobilities. The galactose was also shown to have mobilities identical to those of authentic galactose. The amount of oligosaccharides was too small to permit separation. Hydrolysis after esterification and reduction of the mixture of oligosaccharides gave d-glucose (from d-glucuronic acid) (paper chromatography and glucose oxidase) as the major constituent, with xylose, galactose and the fast pink spot also present. From this it may be concluded that glucuronic acid is the major uronic acid, although galacturonic acid might also be present. However, when the glucose and galactose content of a hydrolysate was assayed before and after esterification, reduction and hydrolysis it was found that the quantity of glucose had more than doubled, while the amount of galactose was smaller in the reduced material. A certain amount of loss is to be expected during these operations but it seems unlikely that galacturonic acid is a constituent of the mucilage.

It was considered from their mobilities that the unknown brown and pink spots might be a methylated hexose and pentose respectively. Demethylation of a hydrolysate led to extensive degradation, and although the pink spot persisted it was impossible to be certain that the disappearance of the brown spot was due to demethylation. 6-O-methyl- and 4-O-methyl-galactoses are common constituents of algal galactans, but in solvent (D) the mobilities of standard 6-O- and 4-O-methylgalactoses and the unknown material were different, the latter being considerably faster.

The standard method of assay for 3,6-anhydrogalactose, a constituent of red algal galactans, is the production of a deep pink colour with resorcinol reagent (Yaphe, 1960), which absorbs at 550 nm. It was found that the mucilage gave the deep pink colour with the reagent. At the same time it was also observed that with the resorcinol reagent both xylose and glucuronic acid gave a bluish mauve colour, which also absorbed at 550 nm. To determine the amount of 3,6-anhydrogalactose in the mucilage it was therefore necessary to measure the absorption of a solution containing the quantity of xylose and glucuronic acid it is considered that 5 mg of mucilage would contain, and to subtract this from the absorption produced by 5 mg of mucilage. The absorption of the xylose-glucuronic acid mixture was about 20% of that of the mucilage which was calculated to contain about 3% of 3,6-anhydrogalactose by weight.

The presence of 3,6-anhydrogalactosidic linkages was supported by very mild acid treatment of the mucilage, sufficient to cleave such linkages, but not the normal glycosidic linkage. This led to a small amount of hydrolysis. The major part of the mucilage was precipitated by ethanol from this hydrolysate, but some remained in solution as oligosaccharides. No monosaccharides were detected in this solution, but a long streak from the starting line was obtained on a paper chromatogram. On further hydrolysis this solution was found to contain all the sugars present in the initial material, as did the ethanol-insoluble fraction. From this it appears that a small proportion of 3,6-anhydrogalactose residues are present and cleaved by this mild treatment.

The sulphate, apart from about 1%, was found to be exceedingly alkali- and acid-labile, and failed to yield any additional sugars after removal by alkali. A paper and a g.l.c. chromatogram of a hydrolysate of the desulphated material were almost identical with those of the initial polysaccharide. The 3,6-anhydrogalactose content of the 2
Mucilage production in Rhodella materials was also the same. The alkaline conditions of methylation were found to have removed the sulphate residue. This is unusual in sulphated polysaccharides (Percival & Smestad, 1972). Analysis of the hydrolysates of the methylated material as the methyl glycosides and as the methylated alditol acetates gave peaks (see Table 4) with the retention times of 2,4- and 2,3-di-O-methyl-xyloses (major), 2,3,6- and 2,4,6-tri- and 2,4- and 2,6-di-O-methyl-galactoses. It can be seen from Table 4 that there are a number of unidentified peaks present which are probably due to the 2 unidentified constituents. That with retention time $T_0$ as the alditol acetate gave fragments of mass 161, 131, 117, 101 and 71, characteristic of a 1,5-di-O-acetyl

Table 4. Retention times ($T$) of methylated sugars present (g.l.c.) in the hydrolysates of the methylated mucilage

<table>
<thead>
<tr>
<th>Column 1</th>
<th>Column 2</th>
<th>Column 7</th>
<th>Corresponding to:</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-6; 1-76</td>
<td>0-66; (0-78)</td>
<td>1-15</td>
<td>Xylose 2,3-di-O-methyl (M)</td>
</tr>
<tr>
<td>1-32; 1-96</td>
<td>(0-73); (0-99)</td>
<td>1-94</td>
<td>2,4-di-O-methyl (M)</td>
</tr>
<tr>
<td>3-2; 4-7; 4-3</td>
<td>1-7; 2-2</td>
<td>2-9</td>
<td>Galactose 2,3,6-tri-O-methyl</td>
</tr>
<tr>
<td>4-0; (4-6)</td>
<td>2-0; (2-4)</td>
<td>2-9</td>
<td>2,4,6-tri-O-methyl</td>
</tr>
<tr>
<td>(2-5); 4-2</td>
<td>(1-9)</td>
<td>2-9</td>
<td>2,4-di-O-methyl</td>
</tr>
<tr>
<td>3-8</td>
<td>5-0</td>
<td>3-8</td>
<td>2,6-di-O-methyl</td>
</tr>
<tr>
<td>1-0*; 1-4*</td>
<td>1-0; 1-3*</td>
<td>2-9</td>
<td>Glucuronic acid 2,3,4-tri-O-methyl</td>
</tr>
<tr>
<td>3-5*; 3-1*</td>
<td>1-83*</td>
<td>1-7*</td>
<td>3,4-di-O-methyl (M)</td>
</tr>
<tr>
<td>0-5; 0-74</td>
<td>0-47; 1-0</td>
<td>0-48; 0-9</td>
<td>Unidentified peaks</td>
</tr>
<tr>
<td>1-06; 0-92</td>
<td>1-1*; 1-5*</td>
<td>1-96</td>
<td></td>
</tr>
<tr>
<td>1-22†</td>
<td>0-7</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

M = major peak; figures in brackets indicate incompletely resolved peaks.
* Present only in reduced material.
† Not present in reduced material.

3,4-di-O-methyl-2-deoxypentitol (Bjorndal, Lindberg, Pilotti & Svensson, 1970), indicating the presence of end-group 2-deoxypentose in the polysaccharide. It is considered that this is the substance which gives the fast pink spot on paper chromatograms.

After partial methylation, to render the material soluble in organic solvents, and so make possible reduction of the uronic acid residues with lithium aluminium hydride, it was reduced and remethylated. The derived reduced methylated polysaccharide, which was readily hydrolysed, was analysed as before. It gave the same peaks as before and in addition peaks with the retention times (see Table 4) of tetra-O-methylglucose (small) and 3,4,6-tri-O-methylglucose (large). All the methylated hexoses and pentoses were confirmed by their fragmentation pattern on mass spectra (Bjorndal, Lindberg & Svensson, 1967).

**DISCUSSION**

The dynamics of mucilage secretion in *Rhodella* are similar to those described for *Porphyridium aerugineum* (Ramus, 1972). However, in *Rhodella* the mucilage envelope is about 10 times thicker than in *P. aerugineum* under normal conditions, and although
reduced, is still present when no synthesis is occurring. This may reflect the need for additional protection in *Rhodella* in its abrasive sand environment, compared with the freshwater habitat of *P. aerugineneum*. However, the composition of the mucilage in *Rhodella* is similar to that of *P. aerugineneum* (Ramus, 1972, 1973; Ramus & Groves, 1972; D.A. Rees, personal communication). In both, xylose is the major sugar, together with lesser amounts of uronic acid and galactose. There is more glucose in *P. aerugineneum*, together with minor components tentatively identified (Ramus, 1973) as guluronic acid, galacturonic acid, another pentose and an anhydrohexose, although Rees (personal communication) has tentatively identified the uronic acids as glucuronic acid and 4-O-methyl-glucuronic acid. In *Rhodella*, the major uronic acid has been characterized as glucuronic acid although the presence of a small amount of galacturonic acid has not been completely ruled out. The anhydrohexose reported by Ramus (1973) in *P. aerugineneum* is probably the 3,6-anhydrogalactose found in the present studies on *Rhodella*. Unequivocal characterization of the 2 reducing substances present in the hydrolysates was not carried out, although the faster of these appears to be a 2-deoxypentose and the slower has the properties of a methylated hexose. The histochemical results on the mucilage are in general agreement with the chemical analysis.

The mucilage contains 10% of sulphate by weight. In *P. aerugineneum*, the sulphate content has been estimated as 10% by Rees (personal communication) and 7.6% by Ramus (1972). Inorganic sulphate labelled with $^{35}$SO$_4^{2-}$ was rapidly taken up from the medium in the light, and incorporated into mucilage which was solubilized into the medium. Incorporation of $^{35}$SO$_4^{2-}$ into red algal polysaccharides has previously been shown by Loewus *et al.* (1971) and Ramus & Groves (1972). In the present study the results indicate that the position and conformation of the sulphate residue is different from that in other sulphated algal polysaccharides.

If the sulphate is present as an ethereal sulphate, R—OS—ONa, then on incineration half of the sulphate should be lost since the sodium in the residue would be sufficient to produce only half a molecular proportion of Na$_2$SO$_4$ (Haaas, 1921). The mucilage in the form of the sodium salt was found to give 15% of ash on incineration and analysis of the ash gave 10% of sulphate based on the weight of mucilage incinerated. Unfortunately, the presence of 10% sulphate cannot be regarded as proof that the sulphate is not present as ethereal sulphate, since it is possible that sodium from the sodium uronate has combined with some of the sulphate and retained it in the ash. It does, however, seem very unlikely that the sulphate is present as cyclic sulphate, as then the polysaccharide would yield only 9.7% ash.

Infrared analysis of the mucilage gave spectra which contained no absorption bands at a frequency of 1240 cm$^{-1}$, characteristic of S=O stretching frequency, or at 820-850 cm$^{-1}$, considered to be characteristic of primary and secondary half ester sulphate attached to sugars, and also characteristic of other sulphated algal polysaccharides (Percival & McDowell, 1967). However, the mucilage gave absorption bands at
1200 cm⁻¹, 875 cm⁻¹ and 950 cm⁻¹, all of which disappear on desulphation. It is interesting to note that the infrared spectrum of heparin, in which the iduronic acid is sulphated on C-2, gives absorption bands at 1230 cm⁻¹, 875 cm⁻¹ and 925 cm⁻¹ (Dietrich, 1968). It is tempting therefore to conclude that it is the uronic acid units in *Rhodella* mucilage which are sulphated.

The failure to detect any other sugar on alkali desulphation indicates the absence of trans elimination and epoxide formation from sulphated xylose units which on hydrolysis should yield arabinose and/or xylose (Percival & Wold, 1963). It is possible that the conformation of the *Rhodella* polysaccharide influences the hydrolysis of the epoxide ring and causes preferential cleavage to yield only xylose. Other investigations have shown that where trans elimination is not possible the sulphate is alkali-stable (Percival, 1949).

1,4-linked galactose 3- or 6-sulphates are converted into 3,6-anhydrogalactose on treatment with alkali (Percival, 1949), but in the present studies there was little, if any, change in the 3,6-anhydrogalactose content on desulphation, showing the absence of these two sulphated residues.

Attempts to fractionate the mucilage into more than a single polysaccharide were unsuccessful. This may have been due to the gelling properties of the material even in very dilute solution. However, the results on DE-cellulose indicated the absence of a neutral polymer and the presence of a single acidic molecular species with a narrow molecular weight range. The failure of the ultracentrifuge experiments made further conclusions with regard to this impossible. Nevertheless, the presence, in the hydrolysates, of oligouronic acids containing xylose, galactose and the 2-deoxypentose shows that the glucuronic acid is linked to each of these sugars.

Methylation indicates the presence of long chains of 1,4- and 1,3-linked xylose units, with some 1,4- and 1,3-linked galactose, and 1,2-linked glucuronic acid. Galactose also appears to be present at branch points linked through C-3 and C-6, and through C-3 and C-4. Tentative evidence for end-group 2-deoxypentose is also presented. Since red algal galactans have alternating 1,3- and 1,4-linked galactose residues it is probable that the 3,6-anhydrogalactose is 1,4-linked, but this sugar is degraded on acid hydrolysis and its linkage has not been determined in these studies.

At first sight it might be considered that we have a 1,3- and 1,4-linked xylan (Turvey & Williams, 1970), and a galactan with alternating 1,3-linked galactose and 1,4-linked 3,6-anhydrogalactose, both typical red algal polysaccharides, but if this were so then mild acid hydrolysis should yield free galactose. Furthermore, the presence of xylose, galactose and 2-deoxypentose in the hydrolysate of the mixture of oligouronic acids indicates that all the sugars are linked together in a complex macro-molecular structure.

Turning to the site of mucilage synthesis within the cell, the Golgi complex has been implicated in mucilage and wall secretion on morphological grounds, but as far as is known there is no experimental evidence for this in red algal cells. As in the brown seaweed *Laminaria* (Evans et al. 1973; Evans & Callow, 1974), and in a variety of animal cells (Young, 1973), autoradiographic evidence in the present study suggests that the transfer of sulphate to an acceptor molecule takes place in the Golgi cisternae.
The intracellular localization of the enzymes of sulphate activation and transfer in *Rhodella* are currently being investigated. Following sulphation, the Golgi cisternae round off to form vesicles which transport the mucilage to the plasmalemma, where it is discharged by exocytosis.

Although protein could not be detected histochemically in the encapsulating mucilage, there is 16% of protein in the solubilized mucilage recovered from the culture medium. Fractionation experiments are currently being carried out to investigate whether the protein component is structurally bound to the polysaccharide molecule. The ER and associated vesicles seen around the forming face of the Golgi bodies may be involved in transfer of protein to the Golgi (see Whaley, Dawalder & Kephart, 1971; Callow & Evans, 1974) or providing a source for input of new membrane (see Flickinger, 1969).

Excretion of low-molecular-weight polypeptide material by algal cells grown under normal conditions commonly occurs (Newell, Dalpont & Grant, 1972). In *Rhodella*, ducts from the subplasmalemmal ER are frequently seen fused to the plasmalemma, thus providing an open channel from the interior of the ER to the outside. Occasionally, electron-dense material is seen at the exit of such ducts, and it is possible that this is the origin of the protein detected in the solubilized mucilage. The role of the subplasmalemmal ER is being investigated further using electron-microscopic autoradiography.

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REFERENCES


Mucilage production in Rhodella


(Received 25 February 1974)

Fig. 6. Median section through a cell showing lobes of the peripheral chloroplast (c), one of which is joined to the central pyrenoid (p). The nucleus (n), starch grains (s) and a Golgi body (g) are also visible. × 10000.

Fig. 7. Living cells of Rhodella in a weak suspension of Indian ink showing the extent of the encapsulating mucilage. × 900.

Fig. 8. Part of a cell showing the subplasmalemmal endoplasmic reticulum (arrow heads) in continuity (arrowed) with the endoplasmic reticulum (er) found in association with the forming face of the Golgi body (g). Part of a mitochondrion (m) is also visible. × 25000.
Mucilage production in Rhodella
Fig. 9. Section through a Golgi body showing fibrillar material in cisternae at the maturing face, a mature mucilage vesicle (v1) closely pressed to the maturing face and another (v2) free in the cytoplasm. There are many small vesicles in the region of the forming and lateral faces of the Golgi body. × 57,000.

Fig. 10. Part of the periphery of a cell showing a Golgi body with associated endoplasmic reticulum (er) and a vesicle (v) with fibrillar content passing between the subplasmalemmal network of endoplasmic reticulum (arrow heads) in transit to the plasmalemma. × 40,000.
Fig. 11. Part of a cell incubated in $^{35}$SO$_4^{2-}$ for 0.5 h showing heavy labelling of the Golgi body (g). Chloroplasts (c) and starch grains (s) are also visible. × 30 000.

Figs. 12, 13. Parts of a cell incubated in $^{35}$SO$_4^{2-}$ for 0.5 h and chased for 1.5 h showing silver grains associated with a Golgi body (g), detached vesicles (arrowed), chloroplast (c) and encapsulating mucilage (mm). × 30 000.

Fig. 14. Part of a cell incubated in $^{35}$SO$_4^{2-}$ for 0.5 h and chased for 3 h showing silver grains associated with the chloroplast (c), Golgi body (g), plasmalemmal region and encapsulating mucilage (mm). × 30 000.

Fig. 15. Light-microscope autoradiograph of cells incubated in $^{35}$SO$_4^{2-}$ for 0.5 h and chased for 5 h, showing heavy labelling around the cell peripheries. Embedded in glycol methacrylate, stained with toluidine blue. × 800.