Chemical Structure of Bacterial Capsular Polysaccharides with special reference to Aerobacter Aerogenes.


October 1955.
ABSTRACT OF THESIS

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Title of Thesis  Chemical structure of bacterial capsular polysaccharides with special reference to Aerobacter Aerogenes

The polysaccharide was obtained as a highly viscous, extracellular slime from a non-encapsulated variant of the bacterium Aerobacter Aerogenes.

The polysaccharide, which had a uronic acid content of ca., 26%, proved extremely resistant to chemical methods of breakdown. Prolonged hydrolysis with 98% formic acid liberated D-glucose, L-fucose and galactose, these being present to the extent of 40%, 10% and ca., 1% respectively. The first two sugars were identified by the formation of 1:2:3:4:6-penta-O-acetyl-D-glucopyranose and L-fucose phenylhydrazone respectively but galactose has only been demonstrated chromatographically.

Although the uronic acid was not identified by the formation of crystalline derivatives, from various experiments its nature was indicated to be glucuronic acid.

Graded hydrolysis of the polysaccharide gave a complex mixture of products. Cellulose in ca., 5% yield was obtained and characterised by formation of α-octa-acetyl cellulose, but due to lack of material the other di-, tri- and oligosaccharides liberated could not be investigated other than by hydrolysis to their component sugars.

The methylated polysaccharide (OMe 42.0%) was hydrolysed with methanolic hydrogen chloride and the hydrolysis products separated into an acidic fraction and a neutral fraction by formation of the barium salt.

The products identified by formation of crystalline derivatives in the neutral fraction were 2:3:4:6-tetra-O-methyl-D-glucose; 3:5 di-O-methyl-6-deoxy-L-galactose; 2:3-di-O-methyl-D-glucose; 2-O-methyl-6-deoxy-L-galactose and the products indicated to be present...
in the neutral fraction were 2:3:6-tri-\(\beta\)-methyl-D-glucose; 2:4:6-
tri-\(\beta\)-methyl-D-glucose; 2:6-di-\(\beta\)-methyl-D-glucose; 3:6-di-\(\beta\)-methyl-
D-glucose; 2-\(\beta\)-methyl-D-glucose; 3-\(\beta\)-methyl-D-glucose and L-fucose.

The acidic fraction was a large unhydrolysed residue. An
attempted separation of this fraction on a column of ion-exchange
resin failed.

Periodate oxidation of the polysaccharide showed that the uptake
of periodate was 1.28 moles per anhydroglucose unit and 0.43 moles
formic acid per anhydroglucose unit were liberated.

The complex nature of the polysaccharide is discussed.
INDEX

Introduction ........................................... 1
Pneumococcal polysaccharides ..................... 2
Mycobacterial polysaccharides ..................... 6
Bacterial Cellulose .................................. 10
Dextran .................................................. 12
Polysaccharide from Phydomonas tumefaciens .... 20
Polysaccharide from Neisserie perflava ........... 22
Polysaccharide from Rhizobium radicicolum ....... 23
Polysaccharide from Azotobacter chroococcum .... 25
Levan .................................................... 26
Polysaccharide from Bacillus anthracis .......... 30
Experimental .......................................... 31
Cultural conditions .................................. 33
Acid hydrolyses ....................................... 37
Estimation of the uronic acid content ............ 41
Graded hydrolysis .................................... 45
Borohydride reductions ................................ 62
Periodate oxidation .................................... 68
Methylation of the polysaccharide (1) ........... 72
Methylation of the polysaccharide (2) ........... 79
Methylation of the polysaccharide (3) ........... 81
Hydrolysis of the methylated polysaccharide ..... 84
Methylation of the polysaccharide (4) ........... 96
Hydrolysis of the methylated polysaccharide ..... 97
Discussion ............................................. 134
Bibliography ........................................... 159
Acknowledgements ..................................... 167
INTRODUCTION
Bacteria produce a great variety of polysaccharides. These may be intracellular, exocellular or capsular, they may remain attached to the bacterium or be liberated into the growth medium but in all cases great controversy exists as to their specific function. The intracellular polysaccharides, like the bacterial glycogens, may act as reserve food material. The other polysaccharides may be merely metabolic byproducts or in the case where they are incorporated into the capsule they presumably serve with the other capsular material to protect the bacterium from its environment.

Although the existence of these bacterial polysaccharides was first recognised almost one hundred years ago the inherent difficulties involved in their isolation rendered even more difficult by the crude techniques then available prevented any serious work from being done on them until comparatively recently. Several very early workers did publish some data about work connected with bacterial polysaccharides but the results are of doubtful value.

A tremendous stimulus was given to work in this field by the discovery of Avery and Heidelberger (1) that the type specificity of the pneumococci was due to polysaccharides contained in their mucinous capsule. This specificity, by means of which the pneumococci are
divided into types, is due to structural variations in the capsular polysaccharide molecules. Friedlander (2) in 1883 had noted a mucin-like substance in the cells of *Diplococcus pneumoniae* but it was Avery and Heidelberger's discovery, coming as it did when new methods of isolating and investigating polysaccharide molecules were becoming available, which led directly to the new field of "Immunopolysaccharides" being opened up and a general revival of interest in bacterial polysaccharides. Since that date interest has never waned in such substances but on the contrary is continually on the increase.

More work has been done on pneumococcal polysaccharides than on any other bacterial polysaccharide both from an interest in their chemical structure and from the viewpoint of determining if these polysaccharides could be used in the prophylaxis of pneumococcal pneumonia.

More than seventy different types (3) of pneumococci are known but despite the amount of work done on them, in only a few cases is anything known of the nature of the type-specific polysaccharide present and indeed in only one case, that of the type-specific polysaccharide from pneumococcus Type III, has a structure been proposed. One or two
facts are known about certain characteristics of the other polysaccharides but in many cases not even the component sugars are known so sparse is our knowledge.

The table following (Table 1) shows a few of the monosaccharides liberated on the hydrolysis of the stated pneumococcal polysaccharides:

Table 1

<table>
<thead>
<tr>
<th>Type</th>
<th>Hydrolytic Products</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>D-galacturonic acid (28%); amino-sugar; acetic acid.</td>
<td>4</td>
</tr>
<tr>
<td>11</td>
<td>D-glucose; L-rhamnose (40%); D-glucuronic acid</td>
<td>5</td>
</tr>
<tr>
<td>111</td>
<td>D-glucose; D-glucuronic acid. (1:1)</td>
<td>6</td>
</tr>
<tr>
<td>1V</td>
<td>D-glucose; N-acetylhexosamine</td>
<td>7</td>
</tr>
<tr>
<td>Vlll</td>
<td>D-glucose; D-glucuronic acid (7:2)</td>
<td>6</td>
</tr>
<tr>
<td>XlV</td>
<td>D-galactose; N-acetyl-D-glucosamine</td>
<td>8</td>
</tr>
</tbody>
</table>

Some idea of the structure of these other polysaccharides can be gained by use of the phenomenon of "cross-reaction", that is reacting the antiserum produced by one polysaccharide of known structure with another polysaccharide of unknown structure. If a reaction occurs then the possibility
is that a structural similarity between the two exists. For example it has been shown (9) that dextran cross reacts with pneumococcus Types 11, XII, and XX antisera.

The extent of cross reaction phenomena into which Type III pneumococcus enters and the relative ease of obtaining it in a high state of purity has led to it being studied extensively. It exhibits a "cross-precipitin reaction" with Type VIII anti-pneumococcal serum (10) and hydrolysis of the two polysaccharides yields the same aldobiouronic acid (6). This suggests that this unit might be the structural similarity between the two responsible for the cross-precipitin reaction.

Hotchkiss and Goebel (11) have shown that the methylated aldobiouronic acid on hydrolysis gives 2:3:6-tri-O-methyl-D-glucose and 2:3:4-tri-O-methyl-D-glucuronic acid, whilst reduction of the methyl ester of the methylated polysaccharide followed by hydrolysis (12) gives 2:3:6-tri-O-methyl-D-glucose and a mixture of the α and ω forms of 2:4-di-O-methyl-D-glucose in equal amounts. This shows that in Type III pneumococcus polysaccharide there is a repeating unit with the D-glucose residue linked to C3 of the D-glucuronic acid, this in turn being linked to C4 of
a second glucose unit. Rotational data suggests a $\beta$-linkage. Figure 1 shows the repeating unit.

![Figure 1](image)

Of the other pneumococcal polysaccharides Type 11 has been partially investigated from a structural point of view and 2:4-di-$\alpha$-methyl-$L$-rhamnose, 2:3-di-$\alpha$-methyl-$D$-glucose, 2:3-di-$\alpha$-methyl-$D$-glucuronic acid and 2:3:4-tri-$\alpha$-methyl-$D$-glucuronic acid have been isolated from the hydrolysis of the methylated polysaccharide. (13) The ratio of the above constituents was found to be 7:1:2:1. Thus the polysaccharide possesses a highly branched structure with the $D$-glucose residues present at the branch points. Until more detailed investigations are carried out however no formula can be proposed for this polysaccharide.

Recently an investigation of Type XVII pneumococcal polysaccharide has been carried out (14). Acid hydrolysis revealed the presence of $D$-glucose and $L$-rhamnose which were isolated as $\beta$-pentacetyl glucose and $L$-rhamnose hydrate respectively.
Organically bound phosphorus, to the extent of 3-4\% was also found to be present the ratio of the constituents being glucose:rhamnose:phosphorus - 5:1:1. Very little else has been deduced about the structure of Type XVIII polysaccharide but the authors tentatively suggest that some of the sugar residues may be joined by phosphate linkages.

**Mycobacterial polysaccharides**

Of the two polysaccharides liberated by *Mycobacterium tuberculosis* (human strain) into the culture medium only the antigenic polysaccharide - designated Polysaccharide 11 - has been extensively studied. Kent (15) found that hydrolysis of the methylated polysaccharide gave 3:4:6-tri-O-methyl-D-glucose in almost exclusive yield. Very little di- or tetra-O-methyl sugar was obtained which suggests either a long chain structure with little branching or a closed ring. The former was in better agreement with sedimentation and diffusion data gained on further investigation of the polysaccharide.

Thus the polysaccharide would seem to consist of glucose residues linked through the 1 and 2 positions and in the pyranose form as shown in figure 2.

The same author studied the rates of oxidation
with potassium periodate of α-methyl glucoside, β-methyl gentiobioside, and the dextrans from *Leuconostoc mesenteroides* and *Leuconostoc dextranicum* (16) and points out that the slow rate of oxidation of Polysaccharide II with potassium periodate precludes the possibility of it having a structure wholly like that of dextran.

*Figure 2*

Two polysaccharides have been isolated from the heat killed cells of *M. tuberculosis* (human strain) (17), one has been found to be associated with the somatic part of the cell, whilst the other was shown to be closely associated with the cell lipoids.

The former polysaccharide was methylated (OMe = 39.9%) and on hydrolysis gave methyl 3:5-di-O-methyl-D-arabinofuranoside (21.9%); methyl 3:4:6-tri-O-methyl-D-mannopyranoside (26.6%); methyl 3:4-di-O-methyl-D-mannopyranoside (20.2%); methyl 2:3:4-tri-O-methyl-L-rhamnopyranoside (24.8%) and a dimethylmethyl-2-acetomidohexoside (5.9%). These percentages correspond to a molecular ratio of 5:5:5:4:1.

The high percentage of terminal residues
(2:3:4-tri-O-methyl-L-rhamnopyranose) coupled with the large amount of dimethyl hexose present suggests a highly branched nature and of the several possible structures which the above data could fit one of the more plausible is given in figure 3.

The numerals represent the points of attachment.

**Figure 3**

\[ \begin{array}{c}
R \quad A \quad M \quad N \\
1 \quad 2 \quad 3 \quad x = 4
\end{array} \]

- **R** - L-rhamnopyranose
- **A** - D-arabinofuranose
- **M** - D-mannopyranose
- **N** - acetomidohexose

The glycosidic linkages are temporarily assigned an \( \alpha \)-configuration based on the change of rotation on acid hydrolysis of both the original polysaccharide and the methylated derivative.

A notable feature of the investigation of this polysaccharide was the demonstration for the first time of arabinose occurring in the furanose form in a substance of microbiological origin.

Investigation of the second polysaccharide, that associated with the lipoid complexes, showed it to
have a highly branched structure. Hydrolysis of its methyl ether (OMe = 42.3%) liberated methyl 2:3:5-tri-O-methyl-D-arabofuranoside (12.8%); methyl 3:5-di-O-methyl-D-arabofuranoside (30.3%); methyl 2:3:6-tri-O-methyl-D-galactopyranoside (33.8%); methyl 3:4-di-O-methyl-D-mannopyranoside (14.4%) and a methyl dimethyl-D-glucosaminide (8%). These percentages correspond to a molecular ratio of 2:5:5:2:1.

Of the possible structures which the above data could fit one of the more probable is depicted in figure 4.

The numerals have the same significance as before.

Figure 4

\[ A' - \text{D-arabofuranose} \]
\[ M - \text{D-mannopyranose} \]
\[ G - \text{D-galactopyranose} \]
\[ N - \text{D-glucosamine} \]

Again the linkages are designated α in view of
Bacterial cellulose

The formation of cellulose by bacteria is comparatively rare but Brown (18) in 1886 recognised it as a component of *Acetobacter xylinum*, where it forms a slimy envelope. Since then other organisms have been found to produce cellulose e.g. *Acetobacter pasteurianum*, *Acetobacter rancens*, *Sarcina ventricula* and *Bacterium xylinoides* (19).

Barsha and Hibbert (20) methylated the cellulose obtained from cultures of *A. xylinum*, grown on a glucose containing medium, by treatment of a partially acetylated bacterial cellulose with di-methyl sulphate and sodium hydroxide followed by methyl iodide and silver oxide and obtained an 85% yield of trimethyl cellulose ($\text{OMe} = 44.0\%$). On treatment with methanolic hydrogen chloride this gave a 92% yield of methyl 2:3:6-tri-O-methyl-D-glucoside which in turn yielded 83% of 2:3:6-tri-O-methyl-D-glucose. By comparing these results with those obtained by studies on natural cellulose the authors came to the conclusion that bacterial cellulose is identical with natural cellulose. Later (21) these same two workers studied the cellulose produced by *A. xylinum* grown on a fructose containing medium and found it...
to be identical to the cellulose produced when the bacterium grew on a glucose containing medium.

Recently (22) the water insoluble polysaccharide of the membrane of *Acetobacter acetigenum* has been structurally investigated. Methylation gave a methyl ether having a rotation in chloroform of -5.75°. This agreed well with the values obtained (-5.0°; -4.0°; -8.0°) for various tri-O-methyl celluloses by Hirst, Owen, Peat and Averill (23). Hydrolysis of the methylated polysaccharide gave 2:3:6-tri-O-methyl-D-glucose and 2:3:4:6-tetra-O-methyl-D-glucose in the ratio of 0.88 mole tetramethyl glucose to 1 mole trimethyl glucose. Calculations based on the partition coefficients of these components between chloroform and water gave a chain length of about 600 glucose units. Thus the bacterial polysaccharide was proved to be a cellulose in which the β-D-glucopyranose units were linked through positions 1 and 4 in essentially unbranched chains.

By conductometric titrations (24), X-ray studies (21), and electron microscope investigations (25), (26), (27), (28), further confirmation of the identity of plant and bacterial celluloses has been obtained. Recently, by viscosimetric studies (29) the cellulose from *A. xylinum* has been shown to be
very similar to amylopectin which has led the authors to propose a branched structure for it.

**Dextrans**

For more than a century the term dextran was used as a generic name for carbohydrate slimes originating from the action of micro-organisms on sugar syrups, fermenting vegetables and other foods. The term now applies specifically to glucans produced by certain bacteria growing on a sucrose containing substrate. These bacteria have been found to be restricted to certain strains of chain forming cocci and have been classified accordingly (30). The classification is Family Coccaceae, Tribe Streptococcaceae, Genus Leuconostoc, Species L. mesenteroides and L. dextranicum.

Structure and properties of the dextran produced by L. mesenteroides.

Hibbert and co-workers (31) were the first to investigate the dextran from L. mesenteroides. The fully methylated polysaccharide \((\text{OMe} = 45.5\%)\) was methanolysed and the mixture of methylated D-glucosides fractionated first by Macdonald's procedure (32) involving differential solubility between chloroform and water. The chloroform soluble material (methyl-tri-\(\) and methyl tetra-\(\)-methyl-D-glucosides) was further fractionated by distillation under reduced
pressure. In this way the material was fractionated into 1 part methyl 2:3:4:6-tetra-O-methyl-D-glucoside to 3 parts methyl 2:3:4-tri-O-methyl-D-glucoside to 1 part methyl 2:3-di-O-methyl-D-glucoside though it was pointed out that the fractionation was not quantitative. Based on these results structures were proposed for the dextran.

However Brauns (33) criticised these results on the grounds that (a) the dextran was incompletely methylated, (b) the ratio of tetra- to tri- to di-O-methyl glucosides of 1:3:1 was not conclusive because of the inefficient fractional distillation employed, and (c) the large percentage (18.4%) of material lost during fractionation.

Correspondingly in 1941, Levi, Hawkins and Hibbert (34) reinvestigated the dextran in order to prove conclusively its structure. The fully methylated product (OMe = 45.6% in 71.4% yield) was hydrolysed at 140° with methanolic hydrogen chloride and the mixture of methyl glucosides obtained in 95% yield quantitatively fractionated on a Podbielniak (35) column. All the products were identified and the ratio of tetra- to tri- to di-O-methyl-glucosides was found to be exactly 1:3:1, thus confirming the results obtained earlier.
These results indicate a cross-linkage for every repeating unit of five D-glucose molecules, and 1,6-linkages within each repeating unit. Two possible structures are shown in figures 5 and 6.

**Figure 5**

```
G'—G'—G'—G'—G'
```

Main chain

**Figure 6**

```
G'—G'—G'—G'—G'—G'
```

Main chain

G - D-glucopyranose.

The numerals represent the points of attachment.

It is conceivable however that the side chains may consist of three, two or even one unit with a corresponding lengthening of the primary chains.

Hassid and Barker (36) studied the dextran produced by *Betacoccus arabinosaceous*, Orla Jensen. From 10g. of the fully methylated produce (OMe = 45.0%) they obtained 6.5g. of tri- and tetra-0-methyl-D-glucosides which by the differential solubility method of Bell (37) they fractionated into 2:3:4:6-tetra-0-methyl-D-glucose (0.27g.), methyl 2:3:4-tri-
O-methyl-D-glucoside (3.92g.) and dimethyl glucose (0.29g.).

Stacey and Swift (38) investigated a water soluble gum like dextran synthesised by another strain of *L. mesenteroides* and found that the physical properties differed very considerably from all the dextrans previously investigated. However on methylation and hydrolysis the sugars isolated were 2:3-di-0-methyl-D-glucose, 2:3:4-tri-0-methyl-D-glucose, and 2:3:4:6-tetra-0-methyl-D-glucose in the same ratio as before i.e. 1:3:1.

But on investigating another strain of the same bacterium Stacey et al. found (39) that the principal glucosidic links were \( \alpha^{-1,6} \) as was usual but the branch points involved positions 1 and 3 and not 1 and 4 as previously found. The fully methylated produce (OMe = 43.3\%) was hydrolysed and the products separated on a silica-gel column. These were characterised as 2:3:4:6-tetra-0-methyl-D-glucose, 2:3:4-tri-0-methyl-D-glucose, and 2:4-di-0-methyl-D-glucose in the ratio of 17:57:26 respectively. The amount of tetramethyl ether corresponds to a chain length of about 6 glucose units. Further confirmation of the presence of 1,3 linkages was obtained from partial hydrolysis studies, oxidation with
periodate and infra-red analysis. The periodate oxidation confirmed the chain length of 6 glucose units.

The authors state that the dextran investigated previously may have contained 1,3 linkages and not 1,4 linkages as their present work was concerned with a dextran produced by an organism derived by subculture from the same strain of *L. mesenteroides*. The possible cause of error may have lain in the closeness of the melting point of an impure N-phenyl-2:4-di-O-methyl-D-glucosylamine to N-phenyl-2:3-di-O-methyl-D-glucosylamine, though it is also possible that over the period of five years subculturing a mutation could have taken place and the new dextran was the result of strain selection.

Using the periodate oxidation technique Jeanes and Wilham (40) investigated the structure of 6 dextrans and by their investigations confirmed the results obtained by the methylation studies that dextrans are polymers of β-D-glucopyranose having a pre-dominance of 1,6 linkages and a variable amount of 1,4 linkages at the points of branching. They found that the ratio of 1,4 to 1,6 glycosidically linked anhydropyranose units varied from 1:3 to 1:24 for dextrans from various strains of *L. mesenteroides*. 
Later Abdel-Akher et al. (41) found that after periodate oxidation of a dextran and reduction of the products there still remained about 2 - 2.5% glucose unattacked thus indicating glucose residues linked 1,3 or 1,2 and 4. Lohmar also found this (42).

From physical studies of dextrans and their triacetates Jeanes and Wilham (43) admit the possibility of 1,3 linkages being present and periodate studies on dextrans from still further strains of L. mesenteroides give added confirmation (44).

Electron microscope studies, have given some confirmation to the structure proposed by Hibbert and co-workers (loc. cit.) for dextran. These indicate (45) that the L. mesenteroides dextran may exist as thin threads possessing a diameter of 30-100A. Assuming a side chain length of five glucose units on either side of a main chain this structure would require a width of approximately 50A and a heavily branched structure would therefore appear plausible. But the fact that a crystalline X-ray diffraction pattern obtained is difficult to explain (46).

Structure of the dextran produced by Leuconostoc dextranicum.

Hibbert and co-workers (47) found that the
dextran produced by *L. dextranicum* was quite different from that produced by *L. mesenteroides*. After methylation and hydrolysis the products isolated were methyl 2:3:4-tri-β-methyl-D-glucoside in 90% yield and 10% of unidentified methyl di-β-methyl-D-glucoside. The authors did not report any trace of tetramethyl sugar. This absence of end group led to the postulation that the dextran was a long, linear polymer in which the terminal units of one chain were in chemical union with the corresponding units of a second chain forming a "net-like" structure.

However in 1939 Peat, Schluchterer and Stacey (48) investigated a dextran from a different strain of *L. dextranicum* and reported the finding of 0.23% methyl 2:3:4:6-tetra-β-methyl-D-glucoside. This percentage indicated a chain length for the dextran of not more than 550 units of -D-glucopyranose linked 1,6. The minimum chain length was found to be of the order of 200 units (49).

A somewhat different dextran has been investigated by Daker and Stacey (50) from *Betabacterium Vermiforme* (Ward-Mayer). The methylated polysaccharide (0Me = 44.0%) was hydrolysed and from the hydrolysate 2:3:4-tri-β-methyl-D-glucopyranose was obtained in 90% yield. In addition 2:3:4:6-
tetra-O-methyl-D-glucose was obtained in about 5% yield. The amount of dimethyl glucose isolated was not significant. Thus the chain length of this dextran is only 25 units and therefore quite different from the dextrans previously reported.

Perhaps the best summary of the state of our knowledge of the structure of dextrans comes from the report of Jeanes, Wilham and co-workers (51). In this report they classify dextrans from 96 strains of bacteria and group them into three main classes as shown in Table 2.

Table 2

Carbon atoms of glucopyranose units involved in glucopyranosidic linkages.

<table>
<thead>
<tr>
<th>Cl (or Cl &amp; C6)</th>
<th>Cl (or Cl &amp; C6) &amp; C4 like</th>
<th>Cl (or Cl &amp; C6) &amp; C3 like</th>
<th>No. of dextrans</th>
</tr>
</thead>
<tbody>
<tr>
<td>Designation and percentage of linkages</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Class 1,6</td>
<td>1,4 like</td>
<td>1,3 like</td>
<td></td>
</tr>
<tr>
<td>A 97 - 50</td>
<td>0 - 50</td>
<td>0 - 2</td>
<td>47</td>
</tr>
<tr>
<td>B 95 - 86</td>
<td>0 - 8</td>
<td>3 - 6</td>
<td>15</td>
</tr>
<tr>
<td>C 85 - 50</td>
<td>0 - 36</td>
<td>6</td>
<td>28</td>
</tr>
</tbody>
</table>

Molecular heterogeneous dextrans which have been fractionated into major components belonging in two or more of the other classes respectively.
These workers found that although methylation studies gave no evidence of branching in dextrans from *L. dextranicum* their evidence suggested that the repeating units were small in some dextrans and there was a high proportion of non 1,6 links indicating branching.

Several other organisms produce polysaccharides which can be loosely classified as dextran-like substances and one such polysaccharide is that produced by *Phytomonas tumefaciens*.

By comparing the optical rotation of a polysaccharide from the above organism in cuprammonium hydroxide and water with the optical rotation of several /\-D-glucosides Reeves (52) suggested that the optical activity of methyl 2-0-methyl-\-/\-D-glucoside in water and cuprammonium hydroxide so closely resembled that of the polysaccharide that the latter was composed of glucopyranose units linked through the 2 position. Table 3 shows his findings.

Table 3/
Table 3

<table>
<thead>
<tr>
<th>Substance</th>
<th>Solvent</th>
<th>Optical rotation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>$[\alpha]_{436}$</td>
</tr>
<tr>
<td>Methyl 2-0-methyl-$\beta$-D-glucoside</td>
<td>Cupra</td>
<td>+985</td>
</tr>
<tr>
<td></td>
<td>Water</td>
<td>-69</td>
</tr>
<tr>
<td>&quot; 3-0- &quot; &quot; &quot; &quot;</td>
<td>Cupra</td>
<td>-86</td>
</tr>
<tr>
<td></td>
<td>Water</td>
<td>-46</td>
</tr>
<tr>
<td>&quot; 4-0- &quot; &quot; &quot; &quot;</td>
<td>Cupra</td>
<td>-1008</td>
</tr>
<tr>
<td></td>
<td>Water</td>
<td>-36</td>
</tr>
<tr>
<td>&quot; 6-0- &quot; &quot; &quot; &quot;</td>
<td>Cupra</td>
<td>+161</td>
</tr>
<tr>
<td></td>
<td>Water</td>
<td>-48</td>
</tr>
<tr>
<td>Polysaccharide Phyt. tumefaciens</td>
<td>Cupra</td>
<td>+960</td>
</tr>
<tr>
<td></td>
<td>Water</td>
<td>-23</td>
</tr>
</tbody>
</table>

Following upon the above suggestion Putman et al. (53) attempted to methylate the polysaccharide for a structural investigation. However they were unable to methylate the polysaccharide fully but considered the methoxyl content (39%) to be sufficiently high to attempt the isolation of a trimethyl derivative. This they were successful in doing and isolated 3:4:6-tri-O-methyl-D-glucose. This gives
further proof to the statement of Reeves that some of the glucose residues are linked through 1,2 glucosidic linkages.

A possible segment of the chain is shown in Figure 7.

Figure 7

Although Barker, Bourne and Stacey (54) say that the polysaccharide synthesised from sucrose by the organism Neisseria perflava is a starch type polysaccharide under the rather loose system of classification here used it can also be considered as a dextran like substance.

Methylation by sodium and methyl iodide in liquid ammonia gave a white solid of methoxyl content 45.1%. This was hydrolysed with methanolic hydrogen chloride and the sugars quantitatively separated on chromatograms. They were eluted from these and estimated by the method of Hirst, Hough and Jones (55).
The average of five assays was:

<table>
<thead>
<tr>
<th>Mol. ratio (%) of components.</th>
</tr>
</thead>
<tbody>
<tr>
<td>2:3-di-O-methyl-D-glucose</td>
</tr>
<tr>
<td>2:3:6-tri-O-methyl-D-glucose</td>
</tr>
<tr>
<td>2:3:4:6-tetra-O-methyl-D-glucose</td>
</tr>
</tbody>
</table>

The proportion of tetramethyl glucose is equivalent to a chain length of 11 to 12 units. Treatment of an aqueous solution of the bacterial polysaccharide with thymol did not afford a linear fraction of the amylose type. From other characteristics, blue value, treatment with /3-amylase and isolation of the limit dextrin the authors conclude that the polysaccharide has properties intermediate between those of amylopectin and glycogen, approximating more closely to the latter.

Another acidic polysaccharide somewhat resembling the pneumococcal polysaccharides is the capsular polysaccharide of *Rhizobium radicicolum* investigated by Schluchterer and Stacey (56). The methylated polysaccharide was subjected to acid hydrolysis and its constituent units were found to be 2:3:6-tri-O-methyl-D-glucose, 2:3-di-O-methyl-D-glucose and 2:3-di-O-methyl-D-glucuronic acid in the ratio of 1:1:1. Graded hydrolysis also
liberated the tetramethyl cellobiouronic acid shown in Figure 8.

Thus the minimum trisaccharide repeating unit must be that depicted in Figure 9.

It is supposed that cross linkages exist with similar chains to give a highly complex laminated molecule. From the proposed structure it might be supposed that a cross-precipitin reaction with Type 111 Pneumococcus antiserum could occur and that is indeed the case. *Rhizobium radicicolum*
polysaccharide also enters into cross-precipitin reactions with a mixture of anti-Penumococcus sera of Types VI and XIV.

The same cross-precipitin reactions occur with the polysaccharide from the capsule of Azotobacter chroococcum. Although this polysaccharide was first isolated in 1938 (57) it was not until the introduction and perfection of the new chromatographic techniques that the complex hydrolysis products of the methylated polysaccharide could be separated and identified.

The free sugars were identified as D-glucose, D-galactose and D-glucuronic acid the latter sugar being present to a small extent only.

Methylation proceeded smoothly to give a product having a final methoxyl content of 40% (58). After hydrolysis the products were separated on a cellulose column and found to contain 2:3:6-tri-O-methyl-D-glucose, 2:4:6-tri-O-methyl-D-glucose, 2:3-di-O-methyl-D-glucose, 2:4:6-tri-O-methyl-D-galactose and an acidic fraction.

The acidic portion was found to be an oligosaccharide and on further hydrolysis it gave 2:4:6-tri-O-methyl-D-glucose, 2:3-di-O-methyl-D-glucose and a di-O-methyl-D-glucuronic acid.
containing the 2:3-di-O-methyl derivative.

Thus the molecule is obviously highly branched, the main chain is built up of glucose and galactose residues in the ratio 3:1 with β-linkages. All the galactose residues and some of the glucose residues are linked 1,3, the other glucose residues are linked 1,4 and 1,6.

The authors point out that the 1,3 linkages of the glucose units, the 1,4 links of the glucuronic acid residues and the presence of β-linkages give the molecule a structural resemblance to the Pneumococcus Type III polysaccharide and this probably accounts for the cross serological reaction.

Levan (Bacterial fructosan)

Levans are polymers of D-fructofuranose units joined by 2,6 links and are synthesised by bacteria grown on media containing sucrose or raffinose. Like dextrans they are a nuisance to the food industry where they may cause contamination of foodstuffs during processing. Recently (59) it has been stated that levans have an important effect on the moisture-binding capacity of soils.

Probably the first investigation on these polysaccharides was done by Greig-Smith and Steel (60) in 1902 on the levan from "Bacillus levaniformans".
They found that it gave only "levulose" (fructose) on hydrolysis and differed in important respects from inulin and starch.

Hibbert and co-workers in 1931 began a series of investigations into the structures of levans produced by various bacteria. On hydrolysis of the levan formed by *Bacillus mesentericus* grown on a sucrose containing medium they obtained 97% of the theoretical yield of fructose (61). Methylation gave a product having a methoxyl content of 44.7% which on hydrolysis gave only 1:3:4-tri-0-methylfructose in 92% yield. They compared the rotation of pure levan (-47.6°) with β-methyl-fructopyranoside (+72.1°) and α-methyl fructopyranoside (+45°) and came to the conclusion that the levan was composed of β-fructofuranose residues. The structure shown in Figure 10 was therefore proposed for the levan.

Figure 10
Later (62) the levan from *B. subtilis* grown on a sucrose containing medium was investigated and this gave 99% of the theoretical yield of fructose on hydrolysis. Methylation again gave only 1:3:4-tri-α-methyl-fructose. The levan formed by *B. subtilis* growing on a raffinose containing substrate was proved to be exactly the same as those previously studied (63). The absence of any tetramethyl fructose in all these investigations would seem to suggest that levans are long unbranched chains of fructofuranose molecules.

However in 1934 Challinor, Haworth and Hirst (64) re-investigated the levan produced by *B. mesentericus* grown on a sucrose containing medium and from the methylated polysaccharide isolated 10% of tetramethyl fructofuranose. This was characterised by converting it to a crystalline amide which proved to be identical with the amide of 2:3:4:6-tetra-α-methyl fructuronic acid. Thus the original sugar was 1:3:4:6-tetra-α-methyl-fructofuranose. The weight of end group obtained corresponds to a chain length of 10 to 12 units. Some dimethyl fructose was isolated in these experiments but was not characterised.

The levans formed by *B. megatherium, Bact. pruni*
(Phytomonas pruni), and Bact. prunicola have been investigated (65) and they can be represented as terminated chains of 10 to 12 2,6 linked fructofuranose units. Thus the amount of tetramethyl fructofuranose isolated from B. megatherium was 9% of the weight of methylated levan hydrolysed giving a chain length of 12 to 13 units, from Bact. pruni - 9.6% giving a chain length of 11 to 12 units and from Bact. prunicola - 11% giving a chain length of 10 to 12 units.

The same structure is proposed for the levan formed by Pseudomonas mors-prunorum (66) and the polysaccharides formed by Gram negative organisms found in milk and certain soil bacteria (67).

By the use of glucose oxidase (notatin) Palmer (68) detected the presence of about 1 glucose molecule per 500 derived by hydrolysis of levan from B. subtilis. Following this up Bell and Dedonder (69) re-examined the levans formed by Ps. prunicola and B. subtilis. They isolated 1:3:4:6-tetra-0-methyl-D-fructose, 1:3:4-tri-0-methyl-D-fructose and 3:4-di-0-methyl-D-fructose in the molar ratio of 1:7-8:1 and concluded that the levans were high branched molecules with the predominant linkage of 2,6 and the branching linkage of 2,1.
The same hydrolysis products have been obtained from the methylated levan of *B. polymyxa* but in the ratio of 1:4:0.66. (70). This finding has been criticised by Bell and Dedonder on the bases that the fractions were not pure but they add that despite this the results clearly show that the levan is a highly branched molecule.

**The polysaccharide from *B. anthracis***

A polysaccharide was first isolated from *B. anthracis* in 1939 (71) and was shown to be composed mainly of D-galactose and N-acetyl-D-glucosylamine. Later two polysaccharides were isolated from the same organism (72) and some insight into their structure gained.

The first of these polysaccharides was shown to be a phosphorylated mannann. Methylation and hydrolysis followed by examination of the products on paper chromatograms revealed the presence of a di- and a tri-0-methyl mannose. The latter compound was shown to be 2:3:6-tri-0-methyl-D-mannose by formation of 2:3:6-tri-0-methyl-D-mannosylaniline.

The second polysaccharide corresponded to that isolated in 1939 and was shown to be made up of D-galactose and N-acetyl-D-glucosamine in the molar ratio of 2:1. Attempts to methylate the polysaccharide proved unsuccessful.
EXPERIMENTAL
Notes on reagents and experimental procedure

Methoxyl determination

This was done by the microvolumetric method of Zeisel (73). The apparatus used was a modified version of the original. The material under investigation was heated under reflux with hydrogen iodide to give methyl iodide, this being absorbed in a solution of sodium acetate and bromine. It was converted to methyl bromide and iodine bromide, the latter being oxidised to sodium iodate. The iodate was then estimated by titration with standard sodium thiosulphate solution.

Solvents for paper chromatograms.

The chromatograms were run in a constant temperature (20°) room on Whatman No. 1 chromatography paper. The solvents used were the upper layers of the systems:

Solvent 2. n-butanol/ethanol/water+4:1:5 v/v (75)
Solvent 3. Ethyl acetate/pyridine/water-10:4:3 (76)
Solvent 4. n-butanol/glacial acetic acid/water+4:1:5 (77)
Solvent 5. Ethyl acetate/glacial acetic acid/formic acid/water-18:3:1:4

A saturated solution of aniline oxalate in water was used as the spray (80) and the papers were developed at 120°.

Melting points

All melting points were determined by the use of a Kofler Hot Stage Microscope.
The bacterium, Aerobacter Aerogenes, usually produces a capsule and slime when grown on nutrient media. By a chance variation upon repeated subculture of a standard strain of Aerobacter Aerogenes, Dr. J. F. Wilkinson of the Department of Bacteriology, University of Edinburgh isolated a purely slime forming variant. The slime formed by this variant forms the subject of this thesis.

Cultural Conditions

The first batch of polysaccharide was grown in one litre screw capped bottles each containing 200 c.c. of the following medium:

- $1.0\% \text{ Na}_2\text{HPO}_4$
- $0.3\% \text{ KH}_2\text{PO}_4$
- $0.03\% (\text{NH}_4)_2\text{SO}_4$
- $0.1\% \text{ K}_2\text{SO}_4$
- $0.1\% \text{ NaCl}$
- $1.0\% \text{ glucose}$
- $0.02\% \text{ MgSO}_4\cdot7\text{H}_2\text{O}$
- $0.002\% \text{ CaCl}_2\cdot6\text{H}_2\text{O}$
- $0.00001\% \text{ FeSO}_4$

This was sterilised by steaming and inoculated from a 24 hour culture of the organism. The bottles were then filled with oxygen through sterile plugged tubing for two minutes. During incubation (48 hours)
the bottles were rotated horizontally at 35°.

Improvements in the technique led to the following method of culture which was used for later batches of material. The isolation of the material was the same in all cases.

The medium in which the bacteria were cultured was the same as before. It was made up with distilled water and contained the relatively high sugar and low nitrogen content found by Duguid and Wilkinson (81) to give maximal polysaccharide production. The medium was sterilised by steaming for thirty minutes on three consecutive days.

Five litre quantities of the medium, after sterilisation were inoculated with loopfuls of the organism grown on agar plates so that each loopful was a heavy inoculum. The flasks were then incubated at 37° for two days with continuous oxygenation - the oxygen being passed through sterile plugged tubing.

Isolation

After the requisite incubation period the now highly viscous solution was centrifuged at 20,000 r.p.m. for 20 minutes. Even at this high speed however it was impossible to centrifuge down all the cells due to the extreme viscosity of the solution (approx. 22xH₂O.)
The centrifugate was decanted and to it one volume of acetone was added with continuous stirring. The stringy lengths of precipitated polysaccharide, still highly gelatinous, adhered to the rod and stirring was continued until all the polysaccharide had been collected in this way. The material was then transferred to another beaker and washed with acetone until the gel had been dehydrated and transformed into a greyish, stringy solid. This was broken up and dissolved in the minimum amount of water before being deproteinised by the Sevag method (82).

To each 250 c.c. of polysaccharide solution, anhydrous sodium acetate (10g.) and glacial acetic acid (5 c.c.) were added the whole being then shaken with a mixture of chloroform:n-butanol (5:1; 60 c.c.) for thirty minutes. After this time the emulsions were centrifuged at 2500 r.p.m. and the proteins were found as white gels at the chloroform: water interface. The aqueous layers were withdrawn and shaken with fresh quantities of the chloroform:butanol mixture until little or no protein was found at the interface.

In earlier preparations it had been found that the extreme viscosity of the solutions made shaking
and centrifugation very difficult. However when it was found that the viscosity could be lessened by steaming the solutions for 60 minutes at pH 7 (phosphate buffer to bromothymol blue indicator) without causing any apparent hydrolysis this step was inserted in the isolation procedure to be done before deproteinisation.

To the deproteinised, aqueous centrifugate twice its volume of acetone was added and the solution allowed to stand overnight in the refrigerator to allow complete precipitation. The precipitated polysaccharide was centrifuged down, dissolved in water and dialysed against running tap water for 96 hours after which time the dialysed solution was freeze-dried to give a pure white, spongy solid.

The yield was approximately 1g. freeze-dried polysaccharide per 3.6 litres of culture medium.
Hydrolysis of the polysaccharide and examination of the products by chromatography

The polysaccharide was found to be extremely resistant to hydrolysis using the standard methods; e.g. \( \text{N-H}_2\text{SO}_4 \) at 100° for 24 hours gave only incomplete hydrolysis. More drastic methods therefore had to be used with the consequent increase in degradation.

Using 72\% \text{H}_2\text{SO}_4 (83)

Polysaccharide (12 mg.) was left in contact with 72\% \text{H}_2\text{SO}_4 (0.1 c.c.) for 17 hours at room temperature. The dark brown solution was diluted to 10 c.c. and boiled under reflux for two hours. After neutralising the solution with barium carbonate and removing the solid matter by filtration the filtrate was taken to dryness and extracted with hot ethanol. The alcoholic solution was examined chromatographically and found to contain only glucose and fucose.

Using 98\% formic acid

Polysaccharide (20 mg.) was heated in a sealed tube with 98\% formic acid (2.0 c.c.) for 24 hours at 100° (bath temp.). The formic acid was removed by vacuum distillation, its complete removal being ensured by adding water (4x0.5 c.c.) to the residue and removing the azeotrope by vacuum distillation also. To destroy any formyl esters that may have
been formed the residue was heated under reflux with 2N-H$_2$SO$_4$ (2.0 c.c.) for four hours on a boiling water bath. The acid was neutralised with barium carbonate, the barium sulphate and excess barium carbonate were filtered off and the solution was deionised by shaking with Amberlite 1R-100(H) resin followed by Amberlite 1R-4B(OH) resin. The solution was concentrated to small volume and examined by paper chromatography. Fucose, glucose and galactose were found to be present.

Previous attempts at hydrolysis using 98% formic acid for shorter times proved ineffective in that hydrolysis was incomplete.

A further hydrolysis was carried out as above except that the solution was not deionised. After neutralising, the barium carbonate and barium sulphate were filtered, methyl alcohol (4 volumes) was added to the filtrate and the solution allowed to stand in the refrigerator overnight. The precipitate so formed was removed at the centrifuge, dissolved in water, the solution deionised by 1R-120(H) resin and the filtrate examined by paper chromatography. Acidic material was shown to be present.

The solution left after the precipitation of
the barium salt was taken to dryness and the residue separated into its components on Whatman 3MM paper using solvent 1. On development with aniline oxalate there was a large amount of material held on the starting line, then galactose, glucose and fucose as usual.

The material held on the starting line was eluted with boiling water. The hard glass obtained was examined chromatographically and found to remain on the starting line when the paper was irrigated with neutral solvents but to travel down the paper slightly when acidic solvents were used.

Hydrolysis of this material with $2N-H_2SO_4$ liberated glucose and fucose together with a further unhydrolysed residue.

A further 24 hour formic acid hydrolysis of the material held on the starting line followed by treatment with $N-H_2SO_4$ for four hours liberated galactose, glucose, fucose, glucurone and an unhydrolysed residue.

Hydrolysis of the polysaccharide and estimation of glucose, fucose and galactose by paper chromatography (55)

The polysaccharide (50 mg.) was hydrolysed with 98% formic acid (5.0 c.c.) in a sealed tube, immersed in a boiling water bath, for 24 hours. The acid was removed as previously described.
Rhamnose (32.2 mg.) was added to the residue and the whole heated under reflux at 100° with N-H₂SO₄ (4 c.c.) for four hours. After neutralising the solution with barium carbonate the syrup left by evaporation of the filtrate was spotted on to Whatman 3MM chromatography paper and irrigated for 45 hours with solvent 1. Side strips were cut off the paper, the positions of the sugars determined by developing the strips with aniline oxalate and the corresponding portions of the main paper cut out. These were left under vacuum overnight in the presence of water vapour then eluted with cold water by the method of Laidlaw and Reid (85) into a Quickfit B.24 8" x 1" boiling tube. About 5 c.c. solution were collected in each case.

Sodium metaperiodate (0.25M.; 1.0 c.c.) was added to each tube and the tightly stoppered tubes placed in boiling water for 20 minutes. Around the top of each tube was a lead water coil to condense any vapours. The tubes were then cooled, neutral ethylene glycol (0.3 c.c.) added to destroy any excess periodate and after 10 minutes standing with occasional shaking the formic acid produced by the oxidation was titrated with 0.01N NaOH using screened methyl orange as indicator. A portion of the paper
which did not contain any sugars was eluted and this used as a blank.

Since 1 mol. of hexose produces 5 mols. of formic acid and 1 mol. of deoxy sugar produces 4 mols. of formic acid, from the known weight of rhamnose used and the titres obtained the ratios of the sugars and hence their percentages can be calculated. The following were the percentages found:

D-Glucose 38.3%  D-Galactose 4.4%  L-Fucose 9.4%

D-Galactose and D-glucose are very difficult to separate on a paper chromatogram and although some separation was achieved there was undoubtedly some overlap so the percentages of glucose and galactose quoted are not strictly precise.

Estimation of the Uronic Acid Content

(a) from the equivalent

Polysaccharide (10.39 mg.) was dissolved in water (5 c.c.) and alkali (0.0102N NaOH; 10 c.c.) added. After standing at room temperature for three hours with occasional shaking the excess alkali was determined by titration with dilute sulphuric acid (0.0104N). The equivalent as found was 683.4. This gives the percentage uronic acid content as 25.8.
(b) by decarboxylation (86)

This was determined by Aspinall, Dudman and Wilkinson (loc. cit.) who found the uronic acid content to be circa 28% by this method.

(c) by the method of Kaye and Kent (87)

Standard solutions of D-glucurone were prepared such that 2 c.c. of any solution contained an amount of D-glucurone in the range 0 - 10μmoles. The glucurone used (Corn Products Ltd., New York) was purified by solution in 95% ethanol, the solution treated with charcoal, hot-filtered and allowed to crystallise.

Aliquots of each solution (2 c.c.) were diluted with water (1 c.c.) and mixed with a freshly prepared solution of hydroxylamine (2 c.c. from 2M-hydroxy-lamine hydrochloride and an equal volume of 3.5M sodium hydroxide). After four minutes at room temperature the solutions were treated with hydrochloric acid (3.34M; 1 c.c.) and ferric chloride (0.37M in 0.1M hydrochloric acid; 1 c.c.). The resulting orange-brown solutions were shaken under reduced pressure (12 mm.) for thirty seconds to remove dissolved gases and then transferred to the cells of a Unicam S.P. 500 spectrophotometer. A blank solution was prepared as above but using water.
in place of the D-glucurone solution.

After compensating for the absorption of the blank solution (freshly prepared in each case) the values of \( \log \frac{I_0}{I} \) for the glucurone solutions at \( E_{5050} \) and \( E_{4750} \) were read off.

The following results were obtained:

<table>
<thead>
<tr>
<th>mols glucurone</th>
<th>( E_{5050} )</th>
<th>( E_{4750} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>9.6</td>
<td>0.98</td>
<td>1.04</td>
</tr>
<tr>
<td>7.9</td>
<td>0.76</td>
<td>0.80</td>
</tr>
<tr>
<td>4.8</td>
<td>0.46</td>
<td>0.51</td>
</tr>
<tr>
<td>3.9</td>
<td>0.37</td>
<td>0.41</td>
</tr>
</tbody>
</table>

Using similar conditions Kaye and Kent (loc.; cit.) obtained the following results:

<table>
<thead>
<tr>
<th>mols glucurone</th>
<th>( E_{5050} )</th>
<th>( E_{4750} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>9.2</td>
<td>0.92</td>
<td>0.982</td>
</tr>
<tr>
<td>8.3</td>
<td>0.78</td>
<td>0.822</td>
</tr>
<tr>
<td>7.4</td>
<td>0.705</td>
<td>0.750</td>
</tr>
<tr>
<td>6.9</td>
<td>0.665</td>
<td>0.710</td>
</tr>
<tr>
<td>6.4</td>
<td>0.585</td>
<td>0.630</td>
</tr>
<tr>
<td>5.5</td>
<td>0.510</td>
<td>0.549</td>
</tr>
<tr>
<td>4.6</td>
<td>0.425</td>
<td>0.475</td>
</tr>
<tr>
<td>2.3</td>
<td>0.205</td>
<td>0.235</td>
</tr>
</tbody>
</table>
The two sets of results are compared on Graph 1.
Reducing groups interfere and it was found better to measure the absorption of the glycosides.
The results for the ester of D-glucurone are:

<table>
<thead>
<tr>
<th>μmols D-glucurone</th>
<th>E'5050</th>
</tr>
</thead>
<tbody>
<tr>
<td>8.0</td>
<td>0.670</td>
</tr>
<tr>
<td>6.0</td>
<td>0.570</td>
</tr>
<tr>
<td>4.0</td>
<td>0.425</td>
</tr>
<tr>
<td>2.0</td>
<td>0.225</td>
</tr>
<tr>
<td>1.0</td>
<td>0.075</td>
</tr>
</tbody>
</table>

These are plotted on Graph 2 and this is taken as the standard curve.

Uronic acid content of polysaccharide

(a) Estimation of the total amount of lactone present in the original polysaccharide

Polysaccharide (3.51 mg.) was dissolved in water (2 c.c.) and the reagents added as before. Zero reading was obtained at E_5050.

(b) Polysaccharide methyl ester

Polysaccharide (2.76 mg.) was heated at 100° (bath temp.) with methanolic hydrogen chloride (0.30 c.c.; 1%) in a sealed tube for 30 minutes. After that time the contents of the tube were added to 1.7 c.c. of water, the reagents added as before
and the reading at $E_{5050}$ noted. Reading 0.43.

From Graph 2 this is equivalent to $4.00 \mu$ moles, giving a uronic acid content of the polysaccharide of 25.5%.

**Graded hydrolysis**

**Preliminary experiments**

To see what conditions gave the optimum yield of products which travelled on paper chromatograms in the di- and tri-saccharide region, 20 mg. of polysaccharide were dissolved in 0.5N hydrochloric acid (1.0 c.c.) and heated under reflux on a boiling water bath.

At specific intervals samples were withdrawn and spotted on a paper chromatogram which was then irrigated with solvent 1 for 48 hours. It was found that the maximum yield occurred after only 30 minutes and no observable increase in yield or new product was apparent after nine hours heating. Thirty minutes was therefore chosen as the hydrolysis time to be used in all future work. The chromatograms revealed the presence of fucose, glucose, galactose, a substance running at the same speed as cellobiose and several substances near and on the starting line.
Examination of the suspected disaccharide

Polysaccharide (50 mg.) was heated under reflux at 100° with 0.5N HCl (2.5 c.c.) for thirty minutes. The solution was neutralised with silver carbonate, the filtrate treated with H₂S gas and the silver sulphide removed by filtration. The filtrate was taken to dryness and spotted on to Whatman 3MM paper. After 48 hours irrigation with solvent 1 the side strips were cut off, sprayed with aniline oxalate and the position of the sugar under investigation determined. That part of the main paper containing the sugar was cut out and eluted with cold water (85). The eluate was made up to 100 c.c., 5 c.c. of this solution taken and the reducing power estimated by the method of Somogyi (88).

No standard curves for cellobiose were available but using a standard glucose curve the percentage cellobiose present was estimated as 5.7, and using a standard maltose curve the percentage present was 5.2.

The remaining solution was taken to dryness, hydrolysed with N sulphuric acid, neutralised and the filtrate examined. Glucose was the only sugar present.
Large scale hydrolysis

Polysaccharide (2.0 g.) was heated under reflux with 0.5N sulphuric acid (100 c.c.) for 30 minutes at 100° (bath temp.). After the required hydrolysis time the solution was neutralised with barium carbonate and the filtrate reduced in volume to 10 c.c.

An equal volume of acetone was added to the solution and the precipitate so produced removed at the centrifuge. (Ppt. GH₁.) Further acetone was added to the centrifugate until it was 70% w.r.t. acetone and this precipitate also centrifuged.

During centrifugation however the precipitate reverted to a glassy fluid. This was not recognised as such at first and some was lost. The fluid that was eventually collected, by decantation, was dissolved in water, de-ionised by Amberlite IR-120(H) ion exchange resin and taken to dryness. (Ppt. GH₂.)

Yield. GH₂ = 141.9 mg.
GH₃ = 702.2 mg.

Examination of GH₃

Examination of GH₃ by paper chromatography using solvent 1 showed glucose and galactose to be present, two sugars running slower than these and close together - one running at the same speed as an authentic sample of cellobiose and the other a little
slower—and finally several oligosaccharides.

Accordingly $\text{GH}_3$ (671 mg.) was dissolved in water (4.0 c.c.) and 0.5 c.c. of this solution pipetted on to each of eight sheets of Whatman 3MM paper. These were irrigated for 72 hours with solvent 1 then the positions of the sugars present found by cutting off and developing the side strips.

Numbering the products from the fastest moving on the chromatogram to the slowest, the following were obtained:

$$\text{GH}_4; \text{GH}_5; \text{GH}_6; \text{GH}_7; \text{GH}_8.$$  

$\text{GH}_4$

The strips containing the sugar were suspended from a double surface coil condenser over water being boiled under reduced pressure. In this way the temperature of the eluting water was kept at 50°. This speeded up the process of elution without increasing the danger of destroying the sugar as might have occurred if the water had been boiled under atmospheric pressure.

Wt. of sugar recovered 17 mg.

On paper chromatograms using solvents 1 and 3 this sugar ran at the same speed as an authentic sample of D-glucose.
Preparation of 1:2:3:4:6-penta-0-acetyl-β-D-glucopyranose from GH₄

Anhydrous sodium acetate (25 mg.) was added to acetic anhydride (1 c.c.) and the whole heated on a boiling water bath for five minutes. GH₄ was added and after heating for an hour on the water bath the liquid was poured into water (5 c.c.) in which a small lump of ice was floating. No precipitation occurred. The water was extracted with chloroform and evaporations of the extracts left a small quantity of a brown oil which crystallised after 12 hours. The crystals were separated, dried and their melting point recorded.

M.pt. GH₄ penta-acetate 126-128°
Mixed m.pt. 127-130°

This was recovered from the chromatograms as for GH₄.

Wt. of sugar recovered = 25 mg.

On paper chromatograms, using solvents 1 and 3 this sugar ran at the same speed as an authentic sample of D-galactose.

Attempted preparation of galactose-ethyl-mercaptal (89)

GH₅ (20 mg.) was dissolved at room temperature
in concentrated HCl (1.0 c.c.) and ethyl mercaptan (1.0 c.c.) added. After shaking the mixture for 5 minutes water was added drop by drop but no crystals appeared. The solvents were removed under reduced pressure but again no crystals appeared. The syrup left was treated with acid and ethyl mercaptan once more followed by very careful addition of water but without success.

\( \text{GH}_6 \)

This was recovered from the chromatograms as for \( \text{GH}_4 \).

Wt. of sugar recovered = 33 mg.

Owing to the proximity of \( \text{GH}_7 \) on the chromatograms there was undoubtedly some contamination of this fraction by \( \text{GH}_7 \) and probably vice-versa.

In solvents 1 and 3 this fraction ran at the same speed as an authentic sample of cellobiose. Hydrolysis with 1.0N-HCl revealed the presence of glucose but also of a trace of galactose.

**Preparation of α-octa-acetyl cellobiose from \( \text{GH}_6 \). (90)**

\( \text{GH}_6 \) was dispersed with stirring in formamide (1.5 c.c.) and pyridine (2.6 c.c.) added. The mixture was stirred for one hour. Acetic anhydride (2.0 c.c.) was added 0.2 c.c. every five minutes. The mixture was then stirred overnight.
The solution was poured into ice-water (5 c.c.) but no precipitate formed. The solvents were removed under reduced pressure to small volume and this extracted with chloroform. The chloroform extracts were evaporated leaving a brown oil which crystallised on standing after three days at room temperature. The crystals were washed with 90% alcohol and their melting point recorded.

- M.pt. acetyl derivative of $\text{GH}_6$ 220-222°
- M.pt. authentic sample of $\alpha$-octa-acetyl cellobiose 221-223°
- Mixed m.pt. 220-222°

$\text{GH}_7$

This was recovered as for $\text{GH}_4$.

Yield of material = 26 mg.

Examination of $\text{GH}_7$ in solvent 1 revealed the presence of two components, and in solvent 4 - three components. Hydrolysis with 2N-$\text{H}_2\text{SO}_4$ showed $\text{GH}_7$ to contain both glucose and galactose. In solvent 1 a crescent shaped spot was also present just below the start line.

$\text{GH}_3$

This was recovered as for $\text{GH}_4$.

Yield of material = 533 mg.

A chromatogram using solvent 4 showed some material to travel in this solvent but some was also
Further hydrolysis of GH₃ (10 mg.) with 2N-H₂SO₄ gave the following results:

<table>
<thead>
<tr>
<th>Solvent 1</th>
<th>Solvent 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Material held on start</td>
<td>Acidic material</td>
</tr>
<tr>
<td>Galactose ?</td>
<td>Glucose and possibly galactose</td>
</tr>
<tr>
<td>Glucose</td>
<td>An unknown substance giving a pink spot (faint)</td>
</tr>
<tr>
<td>Fucose</td>
<td>Fucose</td>
</tr>
<tr>
<td>Spot opposite D-glucurone</td>
<td>Spot opposite D-glucurone</td>
</tr>
</tbody>
</table>

At this stage precipitate GH₁ was examined. A sample (10 mg.) was heated in a sealed tube at 100° with 2N-H₂SO₄ (2 c.c.) for 7 hours. After BaCO₃ neutralisation and deionising with Amberlite LR-100(H) resin the solution was examined by paper chromatography with the following results:

<table>
<thead>
<tr>
<th>Solvent 1</th>
<th>Solvent 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Material held on start</td>
<td>Acidic material</td>
</tr>
<tr>
<td>Galactose ?</td>
<td>Glucose</td>
</tr>
<tr>
<td>An unknown substance giving a pink spot</td>
<td>Fucose</td>
</tr>
<tr>
<td>Glucose</td>
<td>An unknown substance giving a pink spot</td>
</tr>
<tr>
<td>Fucose</td>
<td></td>
</tr>
<tr>
<td>An unknown substance giving a pink spot</td>
<td></td>
</tr>
</tbody>
</table>

GH₁ and GH₈ appeared to have the same general
composition and were therefore combined. \(2N\cdot H_2SO_4\) (8 c.c.) was added and the solution heated in a sealed tube at 100° for 7 hours, neutralised with BaCO\(_3\) and worked up as previously described. Some degradation did occur as the acid solution was very dark.

Yield of material = 382 mg.

Examination of the material by paper chromatography failed to reveal any trace of glucurone.

The hard glass was dissolved in water (1.0 c.c.) and 0.25 c.c. solution pipetted on to four Whatman 3MM papers. These were irrigated for 36 hours with solvent 1, and the positions of the sugars found by spraying side strips. In all, ten substances were revealed, running at approximately the following distances: - 38 cm; 26.5 cm; 22.5 cm; 16.5 cm; 15.5 cm; 10.5 cm; 7 cm; 5 cm; 2.5 cm; and material held on the start line.

Therefore numbering from the fastest moving on the chromatogram these are designated: - \(GH_9\); \(GH_{10}\); \(GH_{11}\); \(GH_{12}\); \(GH_{13}\); \(GH_{14}\); \(GH_{15}\); \(GH_{16}\); \(GH_{17}\); and \(GH_{18}\) respectively.

**Examination of \(GH_9\)**

This was eluted from the paper by suspension from a spiral condenser over a supply of water.
boiling at atmospheric pressure. The time allowed for elution was 45 minutes.

Yield of material = 18 mg.

In solvents 1 and 3 this ran at the same speed as an authentic sample of L-fucose.

Preparation of toluene-p-sulphonhydrazide (91)

p-Toluene sulphonyl chloride (5 g.) dissolved in benzene (10 c.c.) was added to hydrazine hydrate (100%; 3 g.) dissolved in the minimum amount of water. The solution was cooled in ice-water and after two minutes a mass of white crystals formed. These were filtered and recrystallised from benzene. M.pt. 112°.

A stock solution was made by dissolving toluene-p-sulphonhydrazide (1.4 g.) in methanol (20 c.c.).

Preparation of L-fucose toluene-p-sulphonhydrazone

L-Fucose (30 mg.) dissolved in methanol (2 c.c.) was added to 2 c.c. of the stock solution of toluene-p-sulphonhydrazide and the whole heated under reflux for thirty minutes. After five minutes in a refrigerator white crystals appeared.

Yield 47.8 mg. M.pt. 169-172°

Preparation of L-fucose toluene-p-sulphonhydrazone from GH₉

GH₉ was dissolved in methanol (1 c.c.) and 1 c.c. stock solution toluene-p-sulphonhydrazide was added.
The solution was heated under reflux for thirty minutes then stored in a refrigerator. After two days crystals deposited.

M.pt. product from GH\textsubscript{9} \hspace{1cm} 167-169°

M.pt. authentic L-fucose toluene-p-sulphonhydrazide \hspace{1cm} 169-172°

Mixed m.pt. \hspace{1cm} 167-170°

**Examination of GH\textsubscript{10}**

This was recovered from the chromatograms as for GH\textsubscript{9}.

Yield of material = 76 mg.

In solvents 1 and 3 this ran at the same speed as an authentic sample of D-glucose. A rotation in water gave \([\alpha]^{20}_{D} + 53° (H_2O, c=1.5; \text{eq. value})\) cf. \([\alpha]^{20}_{D} + 52.5° (H_2O \text{ theoretical value})\).

**Preparation of 1\textsubscript{2}:2\textsubscript{3}:4\textsubscript{4}:6\textsubscript{6}-penta-O-acetyl-\beta-D-glucopyranose from GH\textsubscript{10}**

Anhydrous sodium acetate (120 mg.) was added to acetic anhydride (5 c.c.) and the whole heated on a boiling water bath until most of the sodium acetate had dissolved. GH\textsubscript{10} was added and after heating for a further hour the liquid was poured into ice-water. An oil separated. After ten minutes stirring the oil solidified. The solution was filtered and the material recrystallised from alcohol.

M.pt. GH\textsubscript{10} acetate \hspace{1cm} 130-131°
M.p.t. authentic 1:3:3:4:6-penta-
\(O\)-acetyl-\(\beta\)-D-glucopyranose 131-132°
Mixed m.p.t. 130-131°

Examination of GH\(_{11}\)

This was recovered from the chromatograms as for GH\(_9\).

Yield of material = 22 mg.

Examination of this fraction by paper chromatography gave very conflicting results. It was run with standards of D-glucose and D-galactose and in some cases it ran at the same speed as glucose whilst in others it ran at the same speed as galactose.

An attempt was made to prepare galactose-1-methyl-1-phenylhydrazone (92) from GH\(_{11}\) but without success.

Examination of GH\(_{12}\)

This was eluted by slowly dripping hot water (85; 200 c.c.) down the paper.

Yield of material = 17 mg.

In an attempt to crystallise the fraction it was dissolved in water (0.3 c.c.) and methanol (3 c.c.) added. \(n\)-Butanol was dropped in until a cloudiness appeared then the solution heated on a steam bath until flocculation occurred. It was set aside for several weeks (93). The fraction did not crystallise.
Examination by paper chromatography using solvent 3 showed the material to give one elongated spot which ran slightly faster than cellobiose, but examination in solvent 4 revealed the presence of five substances. Hydrolysis with 2N sulphuric acid gave glucose and fucose only.

**Examination of GH_{13}**

This was eluted as for GH_{11}.

Yield of material = 11 mg.

In solvents 1 and 3 this ran at the speed as a standard sample of cellobiose though it gave a more elongated spot.

An attempt to induce crystallisation by Whistler’s method (92) proved unsuccessful.

**Attempted preparation of \( \alpha \)-octa-acetyl cellobiose from GH_{13}**

The material was dispersed in formamide (1 c.c.) and pyridine (1.5 c.c.) added. The solution was stirred for 3 hours then acetic anhydride (1.4 c.c.) was added, 0.2 c.c. every 5 minutes. After overnight stirring the solution was poured on to crushed ice. No crystallisation occurred. The water solution was extracted with chloroform, the extracts evaporated and the brown oil left to crystallise. White feather shaped crystals appeared. These were washed with 90% alcohol, in which however they were soluble.
The alcohol washings were evaporated and the oil seeded with authentic celllobiose octa-acetate. It did not however crystallise.

**Examination of $\text{GH}_{14}$**

This was eluted as for $\text{GH}_{11}$. Yield of material = 20 mg.

On chromatograms $\text{GH}_{14}$ ran slower than celllobiose. After several weeks in a vacuum desiccator over $\text{P}_{2}\text{O}_{5}$ the syrup solidified. A melting point was attempted and the following behaviour noted:

- Frothing 34°
- Fluid 101°
- Molten 112°

Hydrolysis with 2N-sulphuric acid revealed the presence of galactose, glucose and fucose.

**Examination of $\text{GH}_{15}$**

This was eluted as for $\text{GH}_{11}$. Yield of material = 28 mg.

The material was deionised on Amberlite IR-120(H) ion exchange resin. As in the case of $\text{GH}_{14}$ this fraction solidified after several weeks in a vacuum desiccator over $\text{P}_{2}\text{O}_{5}$. The behaviour noted on attempting a melting point was:

- Frothing 152°
- Fluid 160° thereafter the material slowly decomposed.
Hydrolysis with 4N hydrochloric acid revealed the presence of an unhydrolysed residue, glucose, fucose and glucurone with an unknown substance giving a pink spot travelling faster than glucose. The ratio, deduced visually, of the components was approximately glucose 4 parts, unknown 1 part, fucose 2 parts and glucurone 1 part.

GH$_{15}$ was heated under reflux with methanolic hydrogen chloride (1%), neutralised and the filtrate taken to dryness.

The residue was dissolved in water (2 c.c.) and added dropwise to a well stirred solution of potassium borohydride (20 mg.) in water (2 c.c.). After twenty minutes, dilute acetic acid (4N) was added and the solution taken to dryness. Methanol was added to the residue and then removed under reduced pressure. This operation was repeated several times. The residue was then dissolved in water and de-ionised on a column of Amberlite IR-120(H) ion exchange resin. After hydrolysis with 4N hydrochloric acid the solution was examined by paper chromatography. Five substances were present, an unknown substance running about the same speed as cellobiose, galactose, glucose and fucose but no trace of glucurone.
Examination of $\text{GH}_{16}$

This was eluted as for $\text{GH}_{11}$.  
Yield of material $= 27 \text{ mg}$.  
This material was de-ionised and after several weeks in a vacuum desiccator it solidified. The substance darkened at $135^\circ$ and thereafter decomposed.  
Hydrolysis of $\text{GH}_{16}$ with 4N hydrochloric acid revealed the presence of an unhydrolysed residue, glucose, an unknown substance giving a pink spot, fucose and glucurone. The ratio of the last four substances was approximately 5:1:2:1.

After treatment with methanolic hydrogen chloride, reduction with potassium borohydride and examination of the products revealed the presence of the same "disaccharide" (?) as in $\text{GH}_{15}$, glucose and fucose.

Examination of $\text{GH}_{17}$

This was eluted as for $\text{GH}_{11}$.  
Yield of material $= 37 \text{ mg}$.  
This material was de-ionised and after several weeks in a vacuum desiccator it solidified. The material decomposed slowly after browning at $134^\circ$.  
Hydrolysis of $\text{GH}_{17}$ with 4N hydrochloric acid and reduction with potassium borohydride following treatment with methanolic hydrogen chloride, gave
the same products as GH\textsubscript{15} except that after reduction galactose was present only in trace amount.

**Examination of GH\textsubscript{18}**

This was eluted as for GH\textsubscript{11}.

Yield of material = 37 mg.

The material was de-ionised and after several weeks in a vacuum desiccator it solidified. The substance decomposed above 183°.

Hydrolysis of GH\textsubscript{18} with 4N hydrochloric acid and reduction with potassium borohydride following treatment with methanolic hydrogen chloride liberated the same products as GH\textsubscript{17}.

**Methoxyl content of original polysaccharide**

The first batch of polysaccharide received had a methoxyl content of 3.88%; 3.83%; 3.84%. A sample of this material (50 mg.) was dissolved in sodium hydroxide (25 c.c.; 0.1N) and heated under reflux for three hours on a bath of boiling water. Acetic acid was added to pH 6 then excess ethanol until the formation of a gelatinous precipitate ceased. The precipitate was washed with ethanol and ether. A methoxyl determination now gave OMe \# 0.2%

Further batches of polysaccharide received later had no such methoxyl value.
Action of 4N hydrochloric acid on the polysaccharide

Following upon the success of liberating glucurone by the action of 4N hydrochloric acid on the products from the graded hydrolysis of the polysaccharide, the action of this acid was studied on the original polysaccharide.

Samples of the polysaccharide were hydrolysed with the acid in sealed tubes at 100° for \( \frac{1}{2} \) hour; \( \frac{3}{4} \) hour; 1 hour; 2 hours; 3 hours; and 4 hours. In all cases the chromatograms revealed the presence of an unhydrolysed residue, galactose, glucose, fucose and glucurone. The latter was present in only small amount. The amount liberated did not increase with increasing time of hydrolysis.

Methanolysis of polysaccharide followed by potassium borohydride reduction (94)

Polysaccharide (190.6 mg.) was heated under reflux for 24 hours with a solution of methanolic hydrogen chloride (6 c.c.; 2%). After this time there was still an appreciable amount of flocculent matter present so the tube was sealed and placed in a bath of boiling water for four hours. The tube was cooled, the contents neutralised with silver carbonate and the filtrate taken to dryness.

The brilliant, golden-yellow residue was dissolved in water (2 c.c.) and added dropwise to a
well stirred solution of potassium borohydride (200 mg.) in water (1.5 c.c.). After the solution had been added the reduction was allowed to proceed for a further 20 minutes then the excess borohydride destroyed by the addition of acetic acid (4N).

The solution was taken to dryness and successive quantities of methanol added to the residue and distilled off to remove boric acid. The residue after this treatment was dissolved in water and de-ionised by passage through columns of Amberlite IR-120(H) and IR-4B(OH) ion exchange resins, the eluate taken to dryness and the residue dissolved in dilute H₂SO₄ (4 c.c.; 1.0N). This solution was heated under reflux for four hours (bath temp. 100°), cooled, neutralised with barium carbonate and the filtrate de-ionised on columns of ion exchange resins as before.

The residue left after removal of the water was spotted on to Whatman 3MM chromatography paper and the chromatogram irrigated with solvent 1 for 48 hours. Side strips were then cut off the paper, sprayed with aniline oxalate to indicate the positions of the sugars and those parts of the main paper containing the glucose and fucose cut out and eluted with boiling water.
The amount of each sugar present was estimated by the method of Somogyi (88) and the ratio of glucose to fucose calculated.

Glucose:fucose = 5.6:1

**Formic acid hydrolysis of the polysaccharide followed by potassium borohydride reduction**

Two weighed samples of polysaccharide A (18.31 mg.) and B (14.15 mg.) were hydrolysed for 24 hours with formic acid (98%; 2 c.c.) in sealed tubes placed in a bath of boiling water. The tubes were then opened, the solution in each quantitatively transferred to flasks and the formic acid removed by vacuum distillation. Successive quantities of water were added to the residues and the formic acid/water azeotrope removed also by vacuum distillation. Weighed quantities of L-rhamnose were added to the flasks (5.45 mg. to A and 3.70 mg. to B respectively) and the contents heated under reflux for four hours with methanolic hydrogen chloride (5 c.c.; 1%). The solutions were neutralised with silver carbonate and filtered.

One residue (sample A) was dissolved in water (2 c.c.) and added dropwise to a well stirred solution of potassium borohydride (20 mg.) in water (2 c.c.). After 20 minutes the borohydride was destroyed by the addition of dilute acetic acid (4N)
and the solution treated as previously described.

Residues A and B were hydrolysed for 4 hours at 100° (bath temp.) with hydrochloric acid (4 c.c.; 1.0N.), the solutions neutralised and worked up as usual.

The products were spotted on to paper chromatograms and these irrigated with solvent 1 for 48 hours after which time those parts of the papers containing glucose, fucose and rhamnose were eluted. The amounts of each sugar present were estimated by the method of Somogyi and the ratio of glucose to fucose in the unreduced sample and the reduced sample calculated.

Unreduced sample

<table>
<thead>
<tr>
<th>Sugar</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>4.66 mg.</td>
</tr>
<tr>
<td>Fucose</td>
<td>1.18 mg.</td>
</tr>
<tr>
<td>Glucose/Fucose</td>
<td>4:1</td>
</tr>
</tbody>
</table>

Reduced sample

<table>
<thead>
<tr>
<th>Sugar</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>7.94 mg.</td>
</tr>
<tr>
<td>Fucose</td>
<td>1.37 mg.</td>
</tr>
<tr>
<td>Glucose/Fucose</td>
<td>5.8:1</td>
</tr>
</tbody>
</table>

In both cases the ratio of fucose to rhamnose was the same.

Methanolysis of the polysaccharide followed by potassium borohydride reduction and graded hydrolysis of the products

Polysaccharide (50 mg.) was heated at 100° in a sealed tube with methanolic hydrogen chloride
(2 c.c; 7%) for four hours. The solution was neutralised with silver carbonate and the residue left after removal of the solvent treated with potassium borohydride for 20 minutes as previously described. At specific intervals samples were withdrawn, neutralised, de-ionised and examined chromatographically.

In three such experiments galactose, glucose and fucose were present but it was not possible to discern if any di- or trisaccharides were present because of streaking on the chromatograms.

The experiment was repeated by heating the polysaccharide under reflux with 1% methanolic hydrogen chloride, but again streaking occurred masking any disaccharides or trisaccharides possibly present.

Attempted characterisation of the uronic acid liberated on hydrolysis of the polysaccharide by formation of 2,2'-D-saccharo-dibenzimidazole (95)

(a) trial run on D-glucurone

D-Glucurone (200 mg.) was dissolved in water (2 c.c.) and bromine (0.5 c.c.) added. The solution was kept at approximately 40° until it was non-reducing to Fehling's solution, then neutralised with silver carbonate. \( \text{H}_2\text{S} \) gas was passed into the warmed filtrate, the silver sulphide removed and the filtrate taken to dryness.
Yield 152 mg.

The following quantities of material were added to the D-glucosaccharic acid contained in a small test-tube.

D-glucosaccharic acid 152 mg. (0.72mM)
o-phenylene diamine 203 mg. (1.88mM)
hydrochloric acid (conc.) 0.15 c.c. (1.88mM)
syrupy phosphoric acid 0.15 c.c. (1.88mM)
Diethylene glycol 1.0 c.c. -

The tube and its contents were heated to 135° ± 5° for 2 hours, the contents then dissolved in water (3 c.c.), a little charcoal added and the hot solution filtered through Gooch asbestos. To the hot filtrate concentrated ammonia was added until the solution was alkaline. On cooling the solution deposited spherical crystals of 2,2'-D-saccharodibenzimidazole.

M.pt. of crystals 235-238°.

(b) on acidic material liberated by the hydrolysis of the polysaccharide

Polysaccharide (500 mg.) was hydrolysed for 24 hours at 100° with formic acid (90%; 50 c.c.) in a sealed tube. The formic acid was removed as described previously and the residue hydrolysed with sulphuric acid (1.0N; 15 c.c.) for 6 hours at 100° (bath temp.). This solution was neutralised with
barium carbonate and ethanol (4 volume) added to the filtrate. The solution was stored at 0°C overnight in the refrigerator and the precipitate then removed at the centrifuge. After de-ionising, by passage through a column of Amberlite IR-120(H) ion exchange resin, the solution was treated with bromine as previously described.

Yield 16 mg.

The following amounts of material were added to the above material contained in a small tube.

<table>
<thead>
<tr>
<th>Material</th>
<th>Amount</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Material</td>
<td>16 mg.</td>
<td>(0.08mM)</td>
</tr>
<tr>
<td>o-phenylene diamine</td>
<td>22 mg.</td>
<td>(0.21mM)</td>
</tr>
<tr>
<td>hydrochloric acid (conc.)</td>
<td>0.02 c.c.</td>
<td>(0.21mM)</td>
</tr>
<tr>
<td>syrupy phosphoric acid</td>
<td>0.02 c.c.</td>
<td>(0.21mM)</td>
</tr>
<tr>
<td>diethylene glycol</td>
<td>0.07 c.c.</td>
<td>-</td>
</tr>
</tbody>
</table>

The mixture was treated as before and on making the filtrate alkaline an appreciable quantity of brown solid deposited. The melting point of this solid was greater than 310°.

Periodate oxidation of polysaccharide

Formic acid estimation (96)

The polysaccharide (154.35 mg.) was dissolved in potassium chloride solution (60 c.c.; 0.56M) by shaking the mixture in the dark and in an atmosphere of nitrogen. The solution (pH = 7.8) was brought
to pH = 6.25 (97) with hydrochloric acid (0.0448N); 1.052 c.c.) by potentiometric titration using a glass electrode and a Pye mains-operated pH meter.

Sodium metaperiodate (20 c.c.; 0.20M) was added and the solution shaken continuously in the dark. A full blank was run simultaneously on the reagents used but it was found that no correction for the acidity of the reagents was necessary.

At intervals samples (10 c.c.) were withdrawn by pipette from the reaction flask, the latter being continuously agitated to ensure the uniformity of the sample each time. Distilled ethylene glycol (1 c.c.) was added and the mixture shaken in the dark for a further 10 minutes.

Nitrogen was bubbled through the mixture before titration with sodium hydroxide (0.0108N) from a semi-micro burette. The passage of nitrogen was continued throughout the titration which was followed potentiometrically by means of a glass electrode and a Pye mains-operated pH meter. The following results were obtained:-
Table 4

<table>
<thead>
<tr>
<th>Polys. (Weight in mg.)</th>
<th>Time (hrs.)</th>
<th>Titre (c.c.)</th>
<th>$\text{H}_2\text{COOH}$/mole</th>
</tr>
</thead>
<tbody>
<tr>
<td>19.29</td>
<td>114</td>
<td>2.100</td>
<td>0.190</td>
</tr>
<tr>
<td>19.29</td>
<td>137</td>
<td>2.925</td>
<td>0.265</td>
</tr>
<tr>
<td>19.29</td>
<td>161</td>
<td>3.750</td>
<td>0.340</td>
</tr>
<tr>
<td>19.29</td>
<td>209</td>
<td>3.750</td>
<td>0.340</td>
</tr>
</tbody>
</table>

Periodate uptake (84)

The polysaccharide (101.60 mg.) was dissolved in sodium metaperiodate solution (100 c.c.; 0.20M) and the solution shaken continuously in the dark. At intervals samples were withdrawn (2 c.c.) and analysed for periodate by the method of Fleury and Lange (98). Sodium bicarbonate (2 c.c.; satd., soln.), excess sodium arsenite (20 c.c.; 0.1000N) and potassium iodide (2 c.c.; 40%) were added to the solution and after ten minutes with occasional shaking the excess arsenite was determined by back titration with standard iodine solution (0.0203N), using two drops of starch solution (1%) as indicator. The following results were obtained:

Table 5

<table>
<thead>
<tr>
<th>Polys. (Weight in mg.)</th>
<th>Time (hrs.)</th>
<th>moles periodate/mole</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.03</td>
<td>24</td>
<td>1.11</td>
</tr>
<tr>
<td>2.03</td>
<td>53</td>
<td>1.16</td>
</tr>
<tr>
<td>2.03</td>
<td>71</td>
<td>1.28</td>
</tr>
<tr>
<td>2.03</td>
<td>95</td>
<td>1.28</td>
</tr>
<tr>
<td>2.03</td>
<td>124</td>
<td>1.28</td>
</tr>
</tbody>
</table>
Ethylene glycol was added to the fully oxidised residue which was then dialysed against running tap water. After concentration under reduced pressure the residue was hydrolysed by hydrochloric acid (1.0N; 6 hours) and examined chromatographically. Glucose and fucose were present with traces of galactose.
Methylation of the Polysaccharide (99)

Polysaccharide (12 g.) was dispersed in water (200 c.c.) by vigorous stirring for 24 hours. To the extremely viscous mass sodium hydroxide (200 c.c.; 60%) was added and the solution stirred in an atmosphere of nitrogen for a further 24 hours until the polysaccharide was completely dissolved. Dimethyl sulphate (180 c.c.) was dropped in over a period of several hours and the solution left to stir overnight. During this time the reaction flask was cooled in ice-water.

Four further methylations were carried out in a like manner, 200 c.c. of acetone being added before the final methylation to assist solution of the partially methylated product.

After the fifth addition of reagents the acetone was removed by distillation, the flask cooled in ice and 2N-H₂SO₄ (1800 c.c.) added gradually until the solution was at pH 8. Care was taken during the addition to ensure that the temperature of the solution remained below 25°. No separation of methylated material occurred either at this stage, when the solution was heated to 80°, or when the pH of the solution was lowered to 6.

The solution was therefore dialysed in "Cellophane"
bags against running tap water for seven days. To the combined solutions, reduced in volume, sodium hydroxide was added until the final solution was 40% with respect to NaOH. Five more methylations were carried out as before. A gelatinous precipitate which formed was filtered and the filtrate brought to pH 8. This produced only a small amount of a white solid. The filtered solid matter was suspended in water and dialysed against running tap water for seven days, the dialysate evaporated to dryness and the residue dissolved in chloroform. The methylated polysaccharide was precipitated by the addition of light petroleum ether (b.pt. 60-80°) to the chloroform solution.

Because of the extreme solubility of the methylated material in chloroform the filtrate from the tenth methylation was extracted with this solvent and the polysaccharide precipitated as before.

Yield 3.33 g. (OMe = 37.4%)

Subsequent recovery from the chloroform and petroleum ether extracts gave a yellow solid (0.513 g.)

The filtrate from the tenth methylation was dialysed and evaporated to dryness. Only 0.817 g. solid material was recovered.
Further methylation with silver oxide and methyl iodide (100)

The methylated polysaccharide (1 g.; OMe = 37.4%) was dissolved in methyl iodide (50 c.c.) containing methanol (5 c.c.) to aid solution. Silver oxide (2.0 g.) was added and the solution heated under reflux. At half hour intervals further quantities of silver oxide (0.5 g.) were added to a total of 8 g. After one hour's heating the solution was filtered and the silver residues exhaustively extracted with chloroform. The syrup obtained by evaporation of the filtrates was methylated a second time by the above procedure.

Yield 0.8 g. (OMe = 42.1%)

The remaining 2.38 g. were therefore similarly methylated. Further methylation of the combined solids did not raise the methoxyl value.

Hydrolysis of the methylated polysaccharide

Preliminary hydrolyses

(a) Using methanolic hydrogen chloride

A sample (20 mg.) of the methylated polysaccharide
was heated in a sealed tube at 100° for 6 hours with methanolic hydrogen chloride (1 c.c.; 1%). The tube was opened and hydrochloric acid (5 c.c.; 1.0N) added. After heating under reflux for three hours the solution was neutralised with silver carbonate, the silver ions removed with H₂S gas and the filtrate examined by paper chromatography.

The following were the results:

<table>
<thead>
<tr>
<th>Colour of spot</th>
<th>Rₜ value</th>
<th>Amount present</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brown</td>
<td>Held on start</td>
<td>Large</td>
</tr>
<tr>
<td>Brown</td>
<td>0.08</td>
<td>Small</td>
</tr>
<tr>
<td>Red</td>
<td>0.11</td>
<td>Small</td>
</tr>
<tr>
<td>Yellow</td>
<td>0.16</td>
<td>Small</td>
</tr>
<tr>
<td>Brown</td>
<td>0.58</td>
<td>Large</td>
</tr>
<tr>
<td>Red ( )</td>
<td>0.90</td>
<td>Large</td>
</tr>
<tr>
<td>Brown ( )</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Red</td>
<td>1.00</td>
<td>Small</td>
</tr>
</tbody>
</table>

(b) Using 95% Formic acid

Polysaccharide (20 mg.) was heated in a sealed tube with 95% formic acid (1 c.c.) for 7 hours at 100°. After removal of the acid the residue was treated with sulphuric acid (2 c.c.; 1.0N) at 100° for 4 hours. Barium carbonate was added until the
solution was neutral and the filtrate examined by paper chromatography. The following results were obtained:

<table>
<thead>
<tr>
<th>Colour of spot</th>
<th>$R_g$ value</th>
<th>Amount present</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brown</td>
<td>Held on start</td>
<td>Small</td>
</tr>
<tr>
<td>Red</td>
<td>0.10</td>
<td>Small</td>
</tr>
<tr>
<td>Yellow</td>
<td>0.17</td>
<td>Small</td>
</tr>
<tr>
<td>Brown</td>
<td>0.58</td>
<td>Large</td>
</tr>
<tr>
<td>Green</td>
<td>0.78</td>
<td>Large</td>
</tr>
<tr>
<td>Red }</td>
<td>0.88</td>
<td>Large</td>
</tr>
<tr>
<td>Brown)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Red</td>
<td>1.00</td>
<td>Small</td>
</tr>
</tbody>
</table>

From these results the formic acid hydrolysis was adjudged the best and was therefore chosen for the large scale hydrolysis.

**Large scale hydrolysis**

Methylated polysaccharide (2.0 g.) was heated under reflux for 7 hours with formic acid (95%; 12.5 c.c.) at 100°C. After 30 minutes however very considerable charring had occurred. The formic acid was removed and hydrochloric acid (20 c.c.; 1.0N) added. After 3 hours at 100°C the solution was neutralised with silver carbonate, filtered, the
silver ions removed with \( \text{H}_2\text{S} \) gas and the filtrate evaporated to dryness.

Yield 0.56 g. (27.6% theory).

To a solution of the syrup, barium hydroxide (0.31 g. in 10 c.c. water) was added followed by excess purified ethanol until the production of a white flocculent precipitate ceased. The solution was allowed to stand overnight then centrifuged.

Yield 54 mg. (alcohol precipitated fraction)
429 mg. (from centrifugate)

Separation of hydrolysis products

A column of powdered cellulose was prepared according to the method of Hough, Jones and Wadman (101). This was washed with water (1000 c.c.), purified \( \text{n}-\text{butanol} \) (500 c.c.) and the eluting solvent \( \text{n}-\text{butanol}/\text{light petroleum (b.pt. 100-120°)} \) (3/7; v/v) saturated with water.

The syrup (400 mg.) dissolved in a little solvent was added to the top of the column, this then being developed in the usual way. The solvent was contained in a constant head reservoir and the receivers changed at specific intervals by an automatic fraction collector. The ratio of \( \text{n}-\text{butanol} \) to light petroleum was gradually increased until a final mixture of 1:1 was used.
The contents of every tenth tube were evaporated and examined by paper chromatography. In this way six main fractions were recovered from the column but not one of them was pure, in that all of them contained more than one substance.

Because of the extreme effect of the formic acid in the initial hydrolysis, the low yields, the difficulty of separating small amounts of closely related substances work was stopped on the material as the results would have had no structural significance.
Attempted methylation of the polysaccharide in liquid ammonia (102)

Ammonia gas from a cylinder of liquid ammonia was passed through four drying towers filled with soda-lime (1 x 9", 3 x 6") then condensed directly into a Freudenberg flask cooled in a slurry of "Drikold" and acetone. Even after passage through the towers a considerable amount of moisture was present in the liquid.

Polysaccharide (546 mg.) was added to the flask which contained about 50 c.c. of liquid ammonia. Sodium and methyl iodide were added with stirring (paraffin seal stirrer). Each addition of sodium was made in such a manner that the amount of free sodium in the reaction mixture did not exceed the amount that would react in the next ten minutes. About one hour was therefore necessary for each addition of sodium, and after the addition of methyl iodide 1½ hour's stirring was allowed before the next addition of sodium.

Seven additions in all were made, the weights of sodium and the volumes of methyl iodide conforming to the following formula:
Addition

1. 2 atoms sodium per C₆ unit

2. 1.8
3. 1.4
4. 0.8

Molar ratio

\[
\text{CH}_3\text{I} = \frac{1.1}{\text{Na}}
\]

Addition

5. 1.4
6. 1.0
7. 0.8

Molar ratio

\[
\text{CH}_3\text{I} = \frac{1.5}{\text{Na}}
\]

Isolation

Addition

<table>
<thead>
<tr>
<th>Addition</th>
<th>mg. Na (theory)</th>
<th>mg. Na (experimental)</th>
<th>c.c. MeI</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>155.0</td>
<td>162.6</td>
<td>0.74</td>
</tr>
<tr>
<td>2.</td>
<td>139.5</td>
<td>148.9</td>
<td>0.67</td>
</tr>
<tr>
<td>3.</td>
<td>108.5</td>
<td>106.9</td>
<td>0.52</td>
</tr>
<tr>
<td>4.</td>
<td>62.0</td>
<td>58.7</td>
<td>0.30</td>
</tr>
</tbody>
</table>

Isolation

<table>
<thead>
<tr>
<th>Addition</th>
<th>mg. Na (theory)</th>
<th>mg. Na (experimental)</th>
<th>c.c. MeI</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.</td>
<td>108.5</td>
<td>108.5</td>
<td>0.71</td>
</tr>
<tr>
<td>6.</td>
<td>77.5</td>
<td>73.1</td>
<td>0.51</td>
</tr>
<tr>
<td>7.</td>
<td>62.0</td>
<td>64.4</td>
<td>0.41</td>
</tr>
</tbody>
</table>

After the fourth methylation it was found impossible to filter the liquid ammonia so decantation was employed to isolate the material. Similarly after the final addition of sodium and methyl iodide.

This gave 520 mg. produce but no methoxyl groups were introduced.
Methylation of the polysaccharide with thallous hydroxide and methyl iodide (103)

Thallium metal was converted into thallous sulphate by the action of hot, concentrated sulphuric acid (104). To form thallous hydroxide an equivalent amount of barium hydroxide was added to a solution of thallous sulphate, the flask being cooled in ice under "Sofnolite". The supernatant liquor - a solution of thallous hydroxide - was siphoned directly into the methylation flask when required.

Polysaccharide (5 g.) was dissolved in water (1000 c.c.) by vigorous stirring. Thallous hydroxide (21 g. in 700 c.c. water) was added and the solution stirred vigorously under nitrogen for four hours at room temperature. The bulk of the water was removed under reduced pressure (nitrogen 12 mm./35°), the last traces being removed by freeze drying. The solid material was finely powdered and kept in a vacuum desiccator over solid potassium hydroxide for 24 hours.

Methyl iodide (150 c.c.) and anhydrous methanol (10 c.c.) were added and the mixture heated under reflux in the dark, and with the exclusion of moisture for 12 hours. After removal of the solvents (under nitrogen) dry A.R. benzene (75 c.c.) was added to the solid material followed by thallous
ethoxide (2.9 c.c.; 10 g.). The mixture was vigorously shaken for 5 minutes then the benzene distilled off. Methyl iodide (100 c.c.) was added and the solution heated under reflux for 12 hours, observing the usual precautions. After removal of the methyl iodide the solid matter was exhaustively extracted with chloroform.

Yield 2.91 g.

The residue was again treated with thallous ethoxide and further extraction raised the overall yield to 3.38 g. The residue from this methylation was treated with thallous hydroxide followed by thallous ethoxide as before. The overall yield was 4.77 g. (OMe = 31.4%).

The combined solids were subjected once more to the thallous ethoxide treatment.

Yield 4.03 g. (OMe = 35.5%).

It was noted that the methylated material was becoming increasingly insoluble in the benzene required as solvent thereby progressively decreasing the yield. In an attempt to effect complete solution of the polysaccharide a little acetone was added to the benzene but on the addition of the thallous ethoxide a copious, evil smelling precipitate was formed.
The solvents were removed at once and the slurry remaining extracted with chloroform. The chloroform extracts were charcoaled and the filtrate taken to dryness giving a black syrup. (3.54 g.) This was remethylated by the above procedure.

Yield 3.45 g. (OMe = 39.5%).

Further methylation did not raise the methoxyl content.

Fractionation of the syrup

Light petroleum ether was purified by overnight shaking with concentrated sulphuric acid, separating the acid, washing with water, sodium bicarbonate, more water and finally drying over calcium chloride. The petrol was distilled, that fraction boiling between 60° and 65° collected and used for the fractionation.

Chloroform was purified by shaking overnight with water, drying over anhydrous sodium sulphate and distilling.

The syrup was heated under reflux for one hour periods with 100 c.c. portions of the solvent.

<table>
<thead>
<tr>
<th>Parts petrol</th>
<th>Parts chloroform</th>
<th>Time</th>
<th>Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>0</td>
<td>2x1 hour</td>
<td>0.292 g. (Fraction 1)</td>
</tr>
<tr>
<td>90</td>
<td>10</td>
<td>5x1 hour</td>
<td>1.405 g. (Fraction 2)</td>
</tr>
<tr>
<td>80</td>
<td>20</td>
<td>4x1 hour</td>
<td>0.115 g. (Fraction 3)</td>
</tr>
</tbody>
</table>
Extensive charring (?) took place and extraction was stopped since no more syrup was being extracted. Fraction 1 was a yellowish oil (0.292 g.), fraction 2 a mobile ruby red fluid (1.405 g.) and fraction 3 a black syrup (0.115 g.).

**Hydrolysis of fraction 2**

Previous work (small scale; OMe = 42%) on formic acid hydrolyses had shown that this method is successful with the material under question if the concentration of the material to be hydrolysed is kept at about 1% w/v.

Correspondingly formic acid (141 c.c.; 90%) was added to the syrup (1.405 g.) and the whole heated under reflux for 8 hours at 100°, this time having been shown to be sufficient for complete hydrolysis. Rotations were taken on samples of the solution at intervals but after 6 hours the solution became too dark for accurate readings.

\[ [\alpha]_p^{35°} = 35° \text{ (20 mins.)}; \ [\alpha]_p^{12°} = 12° \text{ (4 hours)} \]

After removal of the formic acid, hydrochloric acid (75 c.c.; 0.5N) was added and the solution heated under reflux for 6 hours. The acid was neutralised with silver carbonate and the solids and silver ions removed as before.

The filtrate was neutralised with barium carbonate.
filtered and reduced in volume (5 c.c.). This was extracted overnight with chloroform in all glass liquid/liquid extractor. Removal of the chloroform gave a brown syrup. (486 mg.)

The aqueous layer was deionised on a column of Amberlite 1R-100(H) resin until the eluate gave no positive test for barium. Removal of the water gave 368 mg. product.

Acidic fraction 368 mg.
Neutral sugars 486 mg.

354 mg. % hydrolysis = 60.

Separation of hydrolysis products on a cellulose column.

A column of powered cellulose (75 x 2.5 cm.) was prepared according to the method of Hough, Jones and Wadman. The column was washed with water, n-butanol and solvent (initially 70:30/light petroleum:n-butanol) as before.

The syrup (486 mg.) was dissolved in the minimum quantity of solvent and added to the top of the column. After allowing one hour for the syrup to soak in the constant level reservoir was inverted over the top of the column and the eluate collected in test-tubes changed by the automatic fraction collector described by Hough, Jones and Wadman.
Very little separation was achieved on the column as the following table shows.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Tube No.</th>
<th>Weight</th>
<th>Description of chromatogram.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>25-110</td>
<td>301 mg.</td>
<td>Red spot at $R_g 1.0$ and a red/brown spot at $R_g 0.88$.</td>
</tr>
<tr>
<td>2</td>
<td>111-160</td>
<td>89 mg.</td>
<td>Large red spot in confluence with a large green spot.</td>
</tr>
<tr>
<td>3</td>
<td>161-220</td>
<td>11 mg.</td>
<td>Small green spot in confluence with a red spot.</td>
</tr>
<tr>
<td>4</td>
<td>221-300</td>
<td>5 mg.</td>
<td>Small red spot.</td>
</tr>
<tr>
<td>5</td>
<td>301-390</td>
<td>36 mg.</td>
<td>Large brown spot at $R_g 0.58$</td>
</tr>
</tbody>
</table>

442 mg. % recovery = 91.

The tubes were bulked as above and in all cases after removal of the column solvent the residue was treated with water and filtered through a well washed bed of charcoal. The filtrate was evaporated to dryness and the residue shaken with A.R. acetone containing a little charcoal. The solution was filtered and the material recovered from this was the fraction required.

Examination of the fractions

**Fraction 1**

Examination of this by paper chromatography using solvent 6 showed that this solvent gave a good separation of fraction 1 into its components. A
separation of fraction 1 using a cellulose column with solvent 6 as the developing phase was attempted.

A column of powdered cellulose was prepared as before and washed with water (1000 c.c.), purified n-butanol (500 c.c.), benzene (1000 c.c.) containing 2% ethanol (v/v) and finally with the solvent. As the column was flowing too fast to allow the automatic turntable to be used a "Polythene" tube was attached to the end of the column and this tube slightly closed with a screw clip. The procedure was then as before and examination by paper chromatography of the eluate enabled three fractions to be isolated:

- X1. 316 mg.
- X2. 37 mg.
- X3. 172 mg.

525 mg. % recovery = 175!

After investigation it was found that "Polythene" is soluble to some extent in benzene and the increase in weight was due to dissolved polymer.

By running the syrup on Whatman 3MM paper using solvent 2 as the irrigant it was found that the contaminant moved with the solvent front and if a sufficiently long paper was used a separation of the sugars from the contaminant was possible. The separation was not complete however because the contaminant
streaked somewhat.

Five 3 MM papers were spotted with the syrups, the papers irrigated for 24 hours and the positions of the sugars found by spraying side strips. The portion of the main paper containing the sugar was cut out and the paper eluted in a Soxhlet extractor with water then chloroform.

Fraction XI

Weight of fraction recovered = 36 mg.
Chromatographic examination indicated the presence of a single substance R_g 1.0 which corresponded to 2,3,4,6-tetra-O-methyl-D-glucose.

The fraction was extracted into light petroleum (b.pt. 40-60°) and set aside to crystallise. After 24 hours needle shaped crystals appeared. These were recrystallised from the same solvent.

M.pt. fraction XI 84-86°

M.pt. authentic 2,3,4,6-tetra-O-methyl-D-glucose 85-87°

Mixed m.pt. 84-86°

X-ray powder photographs were taken of the unknown and of tetra-methyl glucose. On comparison they were superimposable.

Fraction XII

Weight of fraction recovered = 7 mg.
Chromatographic examination indicated the presence
of a single substance corresponding to 2,3,4,6-tetra-
O-methyl-D-glucose.

Fraction X3

Weight of material recovered = 188 mg.

OMe = 40.8% (cf. OMe = 41.9% for a trimethyl hexose)

Examination of the material by paper chromatography revealed the presence of two substances—though these were not separable by this means. X3 ran at the same speed as an authentic mixture of 2,3,6- and 2,4,6-tri-O-methyl-D-glucose, and gave the same colour of spot—red on top and brown underneath.

Demethylation of X3 (105)

X3 (7 mg.) was heated in a sealed tube with hydrobromic acid (1 c.c.; 48% w/w) at 100° for 5 minutes. The tube was then cooled, opened and the contents diluted to 10 c.c. This solution was neutralised with silver carbonate as before. Examination of the filtrate revealed the presence of glucose only.

Rotation in methanolic hydrogen chloride

X3 (36 mg.) was dissolved in methanolic hydrogen chloride (1%; 2 c.c.) and the rotation observed over a period of 24 hours.
\[ \alpha^{20}_D = +71^\circ \text{ (initial)} \]
\[ +66^\circ \text{ (50 mins.)} \]
\[ +59^\circ \text{ (1.5 hrs.)} \]
\[ +49^\circ \text{ (2.75 hrs.)} \]
\[ +39^\circ \text{ (4.5 hrs.)} \]
\[ +9^\circ \text{ (24 hrs. constant)} \]

Using similar conditions Barker, Hirst and Jones (106) found that 2,3,6-tri-O-methyl-D-glucose showed the following rotational changes:

\[ \alpha^{20}_D = +73^\circ \text{ (initial)} \rightarrow -31^\circ \text{ (min. value)} \]

a fall in rotation of 104°. From the magnitude of the rotational change in X3 the approximate content of 2,3,6-tri-O-methyl-D-glucose is 59%. The solution was neutralised with silver carbonate and the sugars separated on Whatman 3MM paper using solvent 2 as irrigant for 24 hours. The glucofuranoside was hydrolysed with hydrochloric acid (1;ON).

On paper chromatograms this fraction ran at the same speed as authentic 2,3,6-tri-O-methyl-D-glucose and quite distinct from the 2,3,4- and 2,4,6-derivative.

The slower moving fraction ran at the same speed as 2,4,6-tri-O-methyl-D-glucose and quite distinct from the 2,3,6- and 2,3,4- derivative.

**Fraction 2**

This solidified immediately but under the microscope it was seen that syrup surrounded the
crystals which were there and no definite melting point could be obtained.

Paper chromatography indicated fraction 2 to be a mixture of 2:3:6- and 2:4:6-tri-0-methyl-D-glucoses and a substance which gave a green spot on development with aniline oxalate. Estimation of the amount there from the chromatograms gave a result of 3 parts tri-0-methyl glucose to 2 parts unknown.

The fraction had a methoxyl content of 38.5%.

Two demethylations were carried out using the method of Hough, Jones and Wadman but complete demethylation could not be achieved even allowing the solid to remain in contact with the hydrobromic acid for 7 minutes.

A demethylation using hydriodic acid (107) was therefore carried out. Fraction 2 (5.0 mg.) was heated under reflux at 100° with hydriodic acid (2 c.c.) for 10 minutes. The solution was diluted to 15 c.c. and neutralised with silver carbonate. Examination of the filtrate revealed the presence of glucose and fucose.

Fraction 2 was examined further by paper chromatography running it against standards of 2:3:6-tri-0-methyl-D-glucose and 3:4-di-0-methyl-L-fucose. Solvent 2 was used and the time allowed for irrigation
was 17 hours. The unknown ran faster than the di-\(\beta\)-methyl fucose and slightly slower than the tri-\(\beta\)-methyl glucose.

All attempts to separate this fraction into its components using paper chromatography and a great variety of solvent systems met with no success.

**Fraction 3**

All attempts to separate fraction 3 into its components met with no success.

**Fraction 4**

As this fraction was present in only trace amount it was not investigated further.

**Fraction 5**

This had a methoxyl content of 26.8% which seemed to indicate a possible mixture of mono- and dimethyl sugars.

A sample was demethylated by the method of Hough, Jones and Wadman and examination by paper chromatography revealed the presence of glucose and fucose. As before all attempts to separate this fraction into its components failed.

**Acidic fraction**

This fraction had a methoxyl value of 25.4%.

Reduction with lithium aluminium hydride (1) (108)

A portion of the material (50 mg.) was dissolved
in methanolic hydrogen chloride (20 c.c.; 2\%) and heated under reflux for 10 hours. After neutralising the solution with silver carbonate, the syrup obtained by evaporating the filtrate to dryness was dissolved in freshly distilled dioxan (10 c.c.) and added to a suspension of lithium aluminium hydride (50 mg.) in dioxan (10 c.c.). The mixture was stirred (mercury seal) for 3 hours at 32°C. After thoroughly cooling the flask and its contents in ice the excess lithium aluminium hydride was decomposed by the slow addition of water and when decomposition was complete the solution was acidified with dilute H\textsubscript{2}SO\textsubscript{4}. This was taken to small volume leaving a brown oil which was extracted with chloroform. The residue obtained by evaporation of the chloroform extracts was hydrolysed with hydrochloric acid. Chromatographic examination of the filtrate failed to reveal the presence of any sugars.

The chloroform extracted oil was dissolved in methanol, deionised (Amberlite 1R-100(H) and 1R-4B (OH)) and hydrolysed as above. Examination by paper chromatography showed a substance to be present which gave a brown spot at R\textsubscript{G} 0.53 and which ran just slightly slower than standard 2,3-di-\textsubscript{O}-methyl-D-glucose.
Lithium aluminium hydride reduction (2) (109)

A further portion of the acidic material (150 mg.) was heated under reflux for 6 hours with methanolic hydrogen chloride (2%; 1 c.c.) the product dissolved in purified tetrahydrofuran (2 c.c.) and added slowly to a suspension of lithium aluminium hydride (150 mg.) in tetrahydrofuran (2 c.c.). After stirring at room temperature for 4½ hours the complex was destroyed by slowly adding water. Sufficient H₂SO₄ (2N) was added to make the solution just acid then barium hydroxide until the solution was strongly alkaline. The alkaline solution was cooled in ice, centrifuged at 0° and "Drikold" added to the centrifugate until all the carbonates were precipitated and the solution was neutral. The filtrate was taken to dryness and the residue heated under reflux with HCl (1.0N; 5 c.c.) for 6 hours. Silver carbonate was added until the solution was neutral and the filtrate examined.

Two substances were seen to be present, the faster moving one running slightly slower than 2,3-di-O-methyl-glucose on a paper chromatogram. The syrup was separated on Whatman 3MM paper using solvent 2 but on development with aniline oxalate four substances were found to be present having Rₘ
values of 0.86; 0.57; 0.30; and 0.21. As the yields were very small, further investigation was impossible.

Attempted formation of 2:3-di-O-methyl-D-glucaric methyl ester 1,4 lactone and 2:3-di-O-methyl-D-gluconamide.

The acidic material (150 mg.) was dissolved in water (7.5 c.c.) and bromine (1 c.c.) added. The stoppered flask was kept at 37° for 10 days, the bromine removed by aeration and the solution neutralised with silver carbonate. The brown gum left after evaporation of the filtrate was dissolved in methanolic hydrogen chloride (9 c.c.; 2%) and allowed to stand at room temperature for 48 hours. The solution was neutralised and the residue distilled (205°/0.025 mm.). A golden mobile syrup was obtained. The yield was low and extensive charring took place. No crystallisation occurred on long standing or after trituration with various solvents.

To the syrup was added anhydrous methanol (4 c.c.), the solution cooled to 0° and saturated with ammonia gas. After seven days at 0° the solvent was removed. No crystallisation of the residue was apparent after several days. Several more additions of methanolic ammonia were made but all without success.
Second methylation of the polysaccharide with thallous hydroxide and methyl iodide

Polysaccharide (5 g.) was dissolved in water (1000 c.c.) and a solution of thallous hydroxide (500 c.c. water containing approximately 24 g. thallous hydroxide) added. After 4 hours at room temperature with continuous stirring the water was removed (44°/9.5 mm. under N₂). Final traces of water were removed by freeze drying the solid mass. The yellow material was finely powdered and dried over potassium hydroxide in a vacuum desiccator for 24 hours. Neutral methyl iodide (150 c.c.) containing anhydrous methyl alcohol (10 c.c.) was added to the powder and the whole heated under reflux in the dark and with the exclusion of carbon dioxide and moisture for twelve hours. The solvents were removed (30°/9.5 mm. under N₂), the material kept under vacuum until dry then dry A.R. benzene (75 c.c.) and thallous ethoxide (2.9 c.c.; 10 g.) added. After shaking the mixture for five minutes the solvents were removed by vacuum distillation and neutral methyl iodide added (100 c.c.). The solution was heated under reflux for twelve hours with precautions as before, the methyl iodide removed and the solid mass exhaustively extracted with chloroform.

A small yield only was obtained so the mixture
was treated with thallous ethoxide as before. As this did not appreciably increase the yield a further series of methylations was carried out. Then after one more treatment with thallous ethoxide the mass was extracted with chloroform. Yield 3.38 g.

One more complete methylation was carried out and the final yield of methylated product was 3.71 g. of a very dark brown glass (OMe = 39.6%).

Further methylation did not raise the methoxyl content.

Hydrolysis of the methylated polysaccharide

Methanolic hydrogen chloride (200 c.c.; 1%) was added to the methylated material (3.2 g.). Some dissolved but a white precipitate (A) was left. This was filtered and the filtrate (ruby-red in colour) heated under reflux until its specific rotation was constant.

\[
\begin{align*}
[\alpha]^{20}_D & = +35^\circ \text{ (initial)} \\
& +39^\circ \text{ (3½ hrs.)} \\
& +47^\circ \text{ (4½ hrs.)} \\
& +51^\circ \text{ (8½ hrs.)} \\
& +53^\circ \text{ (11 hrs.)} \\
& +53^\circ \text{ (17 hrs. constant)}
\end{align*}
\]

The solution was cooled, neutralised with silver carbonate, the methanol removed from the filtrate and hydrochloric acid (200 c.c.; 0.5N) added to the dark brown syrup producing an immediate brown precipitate (B). After removal of this by filtration the
golden yellow filtrate was heated under reflux (bath temp. 99°) to constant rotation.

Assuming the concentration to be the same i.e. c = 1.5, the following are the specific rotations of the filtrate as determined:

\[
\{\alpha\}^2_{D} = +42^\circ \text{ (initial)} \\
+40^\circ \text{ (1 hr.)} \\
+33^\circ \text{ (6\frac{1}{2} hrs.)} \\
+33^\circ \text{ (10\frac{1}{2} hrs. constant)}
\]

The solution was neutralised with silver carbonate, filtered and \(\text{H}_2\text{S}\) gas passed into the warmed (30°) filtrate to remove any silver ions. The silver sulphide was removed and the filtrate neutralised with barium carbonate which prior to use had been washed several times with water by decantation. The solids were removed by filtration and the filtrate taken to dryness under reduced pressure.

Yield 2.8 g. of a hard, dark brown glass. (D)

The two precipitates (A & B) formed when methanolic hydrogen chloride and 0.5N hydrochloric acid were added to the methylated material were dissolved in methanol, the methanolic solution filtered and taken to dryness. Methanolic hydrogen chloride (50 c.c.; 2%) was added and the solution heated under reflux for 7 hours. The solution was too dark for polarimeter readings to be taken.

Silver carbonate was added until the solution was
neutral, then after filtration and removal of the methanol hydrochloric acid (50 c.c.; 0.5N) was added to the syrup (C) remaining. No solution occurred. The system was heated under reflux for two hours (water bath, 100°C) then the acid decanted into another flask and the hydrolysis continued to a total period of 6½ hours. The solution was cooled, neutralised with silver carbonate and worked up as before. Examination by paper chromatography was negative in that no sugars were present in the residue.

To the syrup (C) left in the flask, after decantation of the acid, methanol was added. Complete solution occurred. The methanol was removed, the syrup dried and methanolic hydrogen chloride (25 c.c.; 7%) added. After heating under reflux for 17½ hours the solution was neutralised with silver carbonate. Hydrochloric acid (25 c.c.; 0.5N) was added but again no solution occurred. As before the acid was decanted and heated under reflux for 8 hours. Chromatographic examination of the hydrolysate failed to reveal the presence of any sugars.

Syrup (C) was hydrolysed with 90% formic acid in a sealed tube at 100° for 9 hours. The formic acid was removed under reduced pressure and water (10 c.c.) added to the residue. No apparent
solution took place. The formic acid - water azeotrope was removed by distillation. Hydrochloric acid (10 c.c.; 0.5N) was added and the solution heated under reflux for 18 hours (bath temp. 98°). After working up as usual the filtrate was examined by paper chromatography. The results revealed three substances to be present; one substance $R_g = 0.83$ corresponded to a synthetic mixture of 2,3,6- and 2,4,6-tri-0-methyl-D-glucose on the chromatogram a second $R_g = 0.63$ corresponded to 2,3-di-0-methyl-D-glucose and the third appeared to be a uronic acid derivative as judged from the cherry-red colour it gave when developed with aniline oxalate.

Separation of the hydrolysis products on a cellulose column

A column of powdered cellulose was prepared as previously described and washed with water (500 c.c.), butanol (500 c.c.), and a mixture of light petroleum (bp. 100/120°) and $n$-butanol in the ratio of 70/30 the whole being saturated with water. All solvents were thoroughly purified before use.

The hydrolysed material (D) being insoluble in 70/30; petrol/$n$-butanol was dissolved in water and pipetted on to the top of the column, allowing each addition to soak in before adding the next. When all the solution had been added it was allowed to
soak in to the cellulose for two hours, after which time the reservoir containing the 70/30; petrol/n-butanol solution was inverted over the column and the automatic fraction collector started.

The contents of every tenth tube were evaporated to small volume on a water bath (100°) and examined by paper chromatography. By this means the separation of the sugars could be found and the tubes containing the various sugars bulked. In some cases as the table below shows, the sugars concerned had $R_g$ values very close to one another and the separation was incomplete. In such cases the contents of every tube near the end of the first fraction and the beginning of the second were examined and so a finer separation achieved, but inevitably there was some overlapping and contamination of one fraction by another.

Table 6

<table>
<thead>
<tr>
<th>Tube No.</th>
<th>Fraction</th>
<th>Solvent used for elution</th>
</tr>
</thead>
<tbody>
<tr>
<td>151-390</td>
<td>1</td>
<td>70/30; petrol/n-butanol/satd $H_2O$</td>
</tr>
<tr>
<td>571-610</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>701-920</td>
<td>3</td>
<td>60/40; petrol/n-butanol/satd $H_2O$</td>
</tr>
<tr>
<td>961-1110</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>1421-1569</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>1691-1790</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>1791-2000</td>
<td>7</td>
<td>from tube 1787, 50/50; petrol/n-butanol/satd $H_2O$</td>
</tr>
<tr>
<td>2891-3050</td>
<td>8</td>
<td>n-butanol/½satd $H_2O$</td>
</tr>
</tbody>
</table>
Before changing the solvent in each case 200 c.c. of the new solvent, unsaturated with water, were allowed to run through the column.

After collecting fraction 8 the column was washed with water (3500 c.c.).

The tubes having been suitably combined, the solvent was removed at 40°/9 mm., the residue dissolved in water and filtered through charcoal to remove any waxy impurities. The solvent was again removed and the residue dissolved in methanol, treated with charcoal, filtered and taken to dryness. The weights of the sugars obtained (weights are the weight of the purified sugar) and the $R_G$ value of the sugar are tabulated below:

Table 7

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Wt. in mg.</th>
<th>$R_G$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>8.0</td>
<td>1.0</td>
</tr>
<tr>
<td>2</td>
<td>5.0</td>
<td>0.87</td>
</tr>
<tr>
<td>3</td>
<td>121.7</td>
<td>0.83</td>
</tr>
<tr>
<td>4</td>
<td>76.5</td>
<td>0.79</td>
</tr>
<tr>
<td>5</td>
<td>45.3</td>
<td>0.60</td>
</tr>
<tr>
<td>6</td>
<td>163.7</td>
<td>0.54</td>
</tr>
<tr>
<td>7</td>
<td>96.9</td>
<td>0.54</td>
</tr>
<tr>
<td>8</td>
<td>190.1</td>
<td>0.20</td>
</tr>
<tr>
<td>water wash (9)</td>
<td>1306.0</td>
<td>-</td>
</tr>
</tbody>
</table>
Examination of the fractions

Fraction 1

Chromatographic examination indicated the presence of a single substance \( (R_g = 1.0) \) which corresponded to 2,3,4,6-tetra-\( \text{O-} \)methyl-D-glucose. The fraction did not crystallise on standing. Its rotation was:

\[
[a]^{20}_D = +87^\circ \quad \text{(equil. value H}_2\text{O. } c = 0.4)
\]

e.f. \[a]^{20}_D = +92^\circ \rightarrow +84^\circ \quad \text{(equil. value H}_2\text{O)} \quad \text{(110)}

Preparation of anilide from fraction 1

Fraction was dissolved in absolute ethanol (2 c.c.), freshly distilled aniline in absolute ethanol (5 mg.; 0.35 c.c.) added and the solution heated under reflux for four hours. After overnight standing at 0\(^\circ\) a small number of crystals separated. These were filtered, washed with dry ether and their melting point recorded.

M.pt. anilide from fraction 1 \quad 123-124^\circ

M.pt. authentic \( (2,3,4,6\text{-tetra-}\text{O-}) \text{methyl-D-glucopyranosyl}-\text{N} \) aniline \quad 128-129^\circ

Mixed m.pt. \quad 125-127^\circ

Fraction 2

This syrup which did not crystallise had an \( R_g \) value of 0.87 and was distinct from fraction 1 and fraction 2 on paper chromatograms.
This fraction had a rotation in water of:

\[ [\alpha]_{D}^{20} = +63^\circ \] (equilibrium value; \( c = 0.5 \))

Its methoxyl content was 41.2\% and demethylation gave only glucose.

For 2:3:4-tri-O-methyl-D-glucose the literature gives the following properties:

\[ [\alpha]_{D}^{20} = +60^\circ \] (equilibrium value; \( c = 1.0;\ H_2O\)).(48)

\[ R_g = 0.85;\ OMe = 41.9\% \]

Fraction 3

Chromatographic examination indicated the presence of two substances which ran at a comparable speed to a synthetic mixture of 2:3:6-tri-O-methyl-D-glucose and 2:4:6-tri-O-methyl-D-glucose. On development with aniline oxalate fraction 3 gave the same characteristic colour as the synthetic mixture viz., red on top and brown underneath.

Demethylation of a portion of this fraction gave only glucose.

The methoxyl value of the fraction was 41.6\% (c.f. OMe 41.9\% for a tri-O-methyl hexose.)

**Rotation of fraction 3 in cold methanolic hydrogen chloride solution**

Fraction 3 (90 mg.) was dissolved in methanolic hydrogen chloride solution (5 c.c.; 2\%) at room temperature and the rotation of the solution observed at intervals.
<table>
<thead>
<tr>
<th>Time (hours)</th>
<th>Observed rotation</th>
<th>$[\alpha]^{18}_D$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>$+1.31^\circ$</td>
<td>$+73^\circ$</td>
</tr>
<tr>
<td>2.5</td>
<td>$+1.18^\circ$</td>
<td>$+66^\circ$</td>
</tr>
<tr>
<td>3.5</td>
<td>$+1.12^\circ$</td>
<td>$+63^\circ$</td>
</tr>
<tr>
<td>5.0</td>
<td>$+1.03^\circ$</td>
<td>$+57^\circ$</td>
</tr>
<tr>
<td>22.0</td>
<td>$+0.66^\circ$</td>
<td>$+37^\circ$</td>
</tr>
<tr>
<td>26.5</td>
<td>$+0.63^\circ$</td>
<td>$+35^\circ$ (constant)</td>
</tr>
</tbody>
</table>

Using similar conditions Barker, Hirst and Jones (loc. cit.) found that 2:3:6-tri-O-methyl-D-glucose underwent the following rotational changes:

$$[\alpha]^{20}_D = +73^\circ \text{ (initial)} \rightarrow -31^\circ \text{ (min. value)}$$

a fall in rotation of $104^\circ$. From the magnitude of the rotational change of fraction 3 the approximate content of 2:3:6-tri-O-methyl-D-glucose present in the fraction is 36%.

No chromatographic solvent could be found which would separate the two isomers satisfactorily. In an attempt to find a suitable alternative method the following experiments were carried out.

**Attempted separation of derivatives of 2:3:6- and 2:4:6-tri-O-methyl-D-glucose**

(a) **Attempted separation of 2:3:6-tri-O-methyl-D-glucono-1,4-lactone and 2:4:6-tri-O-methyl-D-glucono-1,5-lactone**

A synthetic mixture of the trimethyl-D-glucoses (53 mg.) was dissolved in water (4 c.c.) and bromine
(0.5 c.c.) added. The solution was kept at approximately 40° for 24 hours (I11). The excess bromine was then removed by aeration, the solution neutralised with silver carbonate and the syrup obtained from this lactonised by heating it at 87°/0.001 mm. for 60 minutes. Yield 25 mg.

This was spotted on Whatman 3MM chromatography paper and the paper irrigated with the upper layer of water saturated methyl-ethyl-ketone (I12) for 5 hours. The positions of the sugar lactones were found by cutting off the side-strips and spraying them with a solution composed of equal volumes of N-methanolic hydroxylamine hydrochloride and 1.1 N methanolic potassium hydroxide. After ten minutes air drying the strips were sprayed with an aqueous solution containing 1-2% ferric chloride and 1% hydrochloric acid (I13). Only a very fine mist of this spray was allowed to fall on the paper. The sugar lactones showed up as two reddish-brown spots.

The portions of the main paper containing the two substances were cut out and exhaustively extracted with boiling chloroform.

Yield. Slower moving fraction 9 mg. - (Al).
Faster " " 13 mg. - (Bl).
22 mg.
The slower moving fraction was taken to be the 2:4:6-tri-O-methyl-glucono-1,5-lactone and the faster moving fraction 2:3:6-tri-O-methyl-glucono-1,4-lactone.

**Fraction A1**

To this fraction, methanolic ammonia (2 c.c.) was added and the solution allowed to stand at 0° for 48 hours. The solvent was removed but no crystallization occurred. Attempted crystallisations from ether, ether-light petroleum mixtures and ethanol all failed.

**Fraction B1**

This fraction did not crystallise on standing. The material was dissolved in ether and after heating under reflux for four hours two drops of freshly distilled phenylhydrazine were added. After a further four hours heating under reflux the solution was set aside to crystallise. The solvents were removed under reduced pressure and the syrup left triturated with various solvents but without success.

The experiment was repeated on a larger scale and two fractions were obtained - A2 and B2 corresponding to fractions A1 and B1 of the first experiment. With the heavier concentration of material a purple colour was obtained on spraying the paper as opposed to the red-brown colour previously obtained.

**Fraction A2**

Fraction A2 (25 mg.) was dissolved in methanolic
ammonia (2 c.c.) and allowed to stand at 0° for 24 hours. The solvent was removed, methanol added and this allowed to evaporate slowly at room temperature. A few crystals appeared but it was impossible to obtain a melting point from them.

**Fraction B2**

To this fraction (88 mg.) contained in a small tube, five drops of freshly distilled phenylhydrazine and several small lumps of "Drikold" were added (114). The tube was sealed and kept at 120° for 8 hours. After cooling overnight the mass was extracted with ether leaving a light brown solid. This was washed with cold ether in which it seemed insoluble. It was also insoluble in cold benzene and cold ethyl acetate. Boiling ethyl acetate extracted a syrup which failed to crystallise. The melting point of the solid was less than 40° and on exposure to the atmosphere for two or three hours it became syrupy. Cold ether extracted this syrup and left the usual light brown solid which again became syrupy after several hours. The syrup extracted by the ether failed to crystallise.

A further portion of the synthetic mixture of trimethyl-D-glucoses was treated with bromine water until it was non-reducing to Fehlings solution. This gave a hard glass which failed to distil.
(b) by graded elution from a charcoal/celite column (115).

Equal quantities of charcoal (M & B) and celite (Johns-Manville No. 535) were stirred together with concentrated hydrochloric acid for two hours. The mixture was filtered and washed with water until the washings no longer gave an acid reaction. The solid mass was further washed with methyl-ethyl-ketone (500 c.c.) then with a solution of methyl-ethyl-ketone/water (2.5% v/v; 3000 c.c.). A column (16.0 x 1.5 cm.) was prepared of the charcoal/celite mixture and 84 mg. of the synthetic mixture of trimethyl-D-glucoses pipetted on to the top.

Two 500 c.c. flasks A and B were filled to hydrostatic equilibrium, flask A with a 5.5% solution of methyl-ethyl-ketone in water and flask B with a 2.5% solution. Siphon tube C was filled, siphon tube E fitted in to the column through rubber bung G and the siphon started by sucking air out through H and closing tap J. 25 c.c. fractions of the eluate were collected, brought to small volume and examined by paper chromatography using solvent 2.

The sugars came through the column unseparated.

A fresh column was prepared (18.0 x 1.5 cm.) as before, 62 mg. of the mixture pipetted on and the flask filled with a 4% solution of methyl-ethyl-
ketone in water, flask B with pure distilled water. Again no separation occurred.

A fresh mixture of charcoal/celite was prepared and treated only with water. The mixture (100 mg.) was pipetted on to the column prepared from the above charcoal/celite and the column washed with water (1500 c.c.). The water wash was taken to dryness and examined. No sugars were found to be present. Flask A was filled with a 4% solution of methyl-ethyl-ketone in water, flask B with pure, distilled water and the syphons started. Again no separation occurred.

Fraction 4

When examined by paper chromatography this fraction had an \( R_g \) value of 0.79 - 0.80; and always gave a very characteristic green colour when developed with a saturated solution of aniline oxalate, (c.f. fraction 2 of previous methylation). Further examination by paper chromatography showed it to run faster than an authentic sample of 3:4-di-O-methyl-6-deoxy-L-galactose and to run at the same speed as that part of fraction 2 (previous methylation) which gave a green colour with aniline oxalate. A portion (5 mg.) was demethylated by hydriodic acid, the only sugar liberated being fucose.
The methoxyl content of this fraction was 32.2%:
(Calculated for a dimethyl-fucose, $C_8H_{10}O_5 - OMe = 32.3$).

Its rotation was:

$$[\alpha]_{D}^{20} = -72^\circ \text{(initial)} \rightarrow -67^\circ \text{(24 hours, constant. } H_2O. c = 1.5).$$

**Formation of lactone**

A portion of fraction 4 (58 mg.) was dissolved in water (4 c.c.) and bromine (0.5 c.c.) added. After seven days at approximately 40° the bromine was removed from the solution by aeration leaving a brown granular solid. This was removed by filtration. The solution was neutralised with silver carbonate, the solids removed at the centrifuge and $H_2S$ gas passed into the warmed centrifugate until all the dissolved silver had precipitated. The silver sulphide was removed by filtration and the rather cloudy filtrate clarified by treatment with Amberlite IR-120(H) ion-exchange resin.

Yield:

- 33.3 mg. (syrup)
- 11.2 mg. (granular material)
- 44.5 mg.

The syrup was lactonised by heating at 90°/0.05 mm. for one hour. No crystallisation was apparent after several weeks at room temperature or at 0°.
Rotation of lactone

The lactone (33 mg.) was dissolved in water (5 c.c.) and its rotation observed over a period of time.

\[ [\alpha]^{20}_D = +66^\circ \text{ (initial)} \rightarrow +51^\circ \text{ (7 days, constant)} \]

(c = 0.66)

Preparation of amide

The material recovered from the above was relactonised (90°C/0.002 mm.), dissolved in methanolic ammonia (3 c.c.) and allowed to stand at room temperature for 48 hours. The solvent was removed and the syrup allowed to stand for some time but no crystals formed. Attempts at trituration were also unsuccessful.

Weerman reaction on dL-O-methyl fuconamide (116).

Sodium hypochlorite solution was prepared by dissolving sodium hydroxide (10 g.) in water (15 c.c.) and bubbling in chlorine (5.5 g.) from a cylinder of liquid chlorine. The solution was then made up to 100 c.c.

The amide was dissolved in water (1 c.c.), sodium hypochlorite solution added (1 c.c.) and the solution kept at 0°C for 3 hours. The excess hypochlorite was destroyed by addition of sodium thiosulphate, then sodium acetate and semicarbazide hydrochloride (saturated solution) added producing
an immediate, copious, white precipitate which persisted for two minutes then slowly disappeared. The solution was left at 0° and crystals slowly deposited. These were separated, washed and dried.

M.pt. of above crystals 253-256°
M.pt. of an authentic sample of hydrazodicarbonamide 254-256°
M.pt. of a mixture of crystals and authentic sample 252-254°

Formation of methyl glycoside

A portion of fraction 4 (10 mg.) was dissolved in methanolic hydrogen chloride (7 c.c.; 2%) and heated under reflux for 7 hours. After neutralising the solution with silver carbonate and filtration the solvents were removed and the syrup obtained dissolved in dilute hydrochloric acid (0.012N.: 2 c.c.). This solution was heated under reflux (water bath. 100°) and the rate of hydrolysis of the glycoside followed polarimetrically.

<table>
<thead>
<tr>
<th>Time (hours)</th>
<th>$[\alpha]^{20}_D$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>+29°</td>
</tr>
<tr>
<td>0.5</td>
<td>+42°</td>
</tr>
<tr>
<td>1.0</td>
<td>+51°</td>
</tr>
<tr>
<td>1.5</td>
<td>+55°</td>
</tr>
<tr>
<td>2.5</td>
<td>+55° (constant).</td>
</tr>
</tbody>
</table>
Periodate uptake of fraction 4

A portion of fraction 4 (3.25 mg.) was dissolved in water (2 c.c.) and sodium metaperiodate (4 c.c. 0.02M) added. The solution was shaken in the dark for 6 hours. A full blank was run at the same time. After the 6 hours phosphate buffer (4 c.c.) was added to the solution then excess potassium iodide (4 c.c. of a 20% aqueous solution). The solution was shaken for five minutes then titrated with 0.0670N sodium arsenite using three drops of a 1% starch solution as indicator. The following results were obtained:

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Blank titre</td>
<td>1.806 c.c.</td>
</tr>
<tr>
<td>Experimental titre</td>
<td>1.224 c.c.</td>
</tr>
<tr>
<td>Difference</td>
<td>0.582 c.c.</td>
</tr>
</tbody>
</table>

Hence mols. oxidised/mol. = \( \frac{192 \times 0.582 \times 0.0670 \times 1000}{1 \times 1000 \times 3.25 \times \frac{1}{2}} \)

= 1.15

Following upon the success of isolating Fraction 4 in a pure state from a cellulose column, Fraction 2 from the previous hydrolysis of the methylated polysaccharide was re-investigated.

A cellulose column (25 x 1.0 cm.) was prepared as before, the eluting solvent being the upper layer of a water saturated solution of light petroleum (b.p. 100 - 120°)/\( \_n \)-butanol the latter two solvents
being in the ratio of 60:40.

Fraction 2, dissolved in the minimum amount of solvent, was pipetted on to the column and the eluate collected in small test tubes which were changed at specific intervals by an automatic fraction collector.

Tube 31 contained the mixture of tri-_-methyl-D-glucoses and the di-_-methyl-L-fucose, but tubes 32 to 42 (inclusive) contained only the latter sugar.

The contents of the tubes were bulked and the solvents removed leaving a crystalline residue. This was dissolved in water, treated with charcoal, filtered and the solvent removed. The residue then remaining was dissolved in acetone, treated with charcoal, filtered and on removing the acetone white, dendritic crystals formed immediately.

Yield 21 mg. OMe = 32.2% R_G = 0.80 (green spot)

The crystals melted at 118 - 121°. Crystalline 2:3:6-tri-_-methyl-D-glucose (m.p. 122°) depressed the melting point by 33°.

Rotation

\[ [\alpha]^{20}_D = -100^\circ \text{ (initial)} \rightarrow -69^\circ \text{ (24 hours: constant)} \]

\[ (c = 0.42; H_2O) \]

Fraction 5

Chromatographic examination revealed the presence of a single sugar \( R_G = 0.65 \) which ran at
the same speed as an authentic sample of 2:3-di-O-methyl-D-glucose.

Demethylation of a portion of the syrup gave only glucose. The methoxyl value of the fraction was 29.7\% (c.f. OMe = 29.7\% for a di-O-methyl hexose).

Its rotation was:

$$[\alpha]_D^{20} = +64^\circ \text{ (equilibrium value; } c = 0.52; \text{ H}_2\text{O})$$

For 2:3-di-O-methyl-D-glucose the literature gives:

$$[\alpha]_D^{20} = +64.3^\circ \text{ (equilibrium value; } c = 2.0; \text{ H}_2\text{O})$$

The syrup failed to crystallise on standing.

**Preparation of anilide from fraction 5**

A portion of fraction 5 (20 mg.) was dissolved in absolute ethanol (2 c.c.) and freshly distilled aniline (9.3 mg.) in absolute ethanol (0.5 c.c.) added. The solution was heated under reflux for 4 hours and allowed to stand overnight in a refrigerator when white crystals deposited. These were separated, washed and recrystallised from ethanol.

M.pt. of crystals 130-132\°

M.pt. of authentic N-phenyl-2:3-di-O-methyl-D-glucosylamine 129-132\°

M.pt. of a mixture of crystals and authentic sample 129-131\°

**Fraction 6**

This travelled on paper chromatograms as a
single substance but a low methoxyl content (OMe = 23.3%; 23.5%) indicated a possible mixture of mono- and di-methyl sugars. It was found that by addition of acetone to the syrup a fractionation into an acetone soluble and an acetone insoluble portion could be effected. By repeated acetone addition, filtration and solvent removal followed by further acetone addition, fraction 5 was eventually completely fractionated.

Fraction 6 - acetone insoluble material

The weight of acetone insoluble material recovered was 43 mg. Examined by paper chromatography revealed the presence of a single substance which gave a green coloured spot on development with aniline oxalate. Demethylation of a sample of this fraction by the method of Hough, Jones and Wadman (loc. cit.,) showed fucose only to be present.

The methoxyl content of the fraction was 17.5% (c.f. OMe = 17.4% calculated for a monomethyl fucose).

\[ [\alpha]^{20}_D = -71^\circ \text{ (initial)} \rightarrow -81^\circ \text{ (24 hours constant)} \text{ (H}_2\text{O. c = 0.33)} \]

c.f. \[ [\alpha]^{20}_D = -83^\circ \text{ (equilibrium rotation of 2-0-methyl-6-deoxy-L-galactose (107))} \]

An authentic specimen of the above sugar was obtained and compared on paper chromatograms with the material under investigation. In all cases the two
ran at the same speed and a mixture of the two ran as
a single substance.

M.pt. acetone insoluble material 142-145°
M.pt. of an authentic specimen of
2-0-methyl-6-deoxy-L-galactose 147-149°
M.pt. of a mixture of the above two
materials 144-148°

Fraction 6 - acetone soluble fraction

Demethylation of a sample of this material gave
glucose in quantity. From its position on paper
chromatograms and its methoxyl value of 29.6% this
fraction was considered to be a mixture of 2:6- and
3:6-di-0-methyl-D-glucose. An authentic mixture of
these two sugars was obtained and compared on paper
chromatograms with the acetone soluble material
under investigation. Both substances ran at the
same speed and a mixture of the two ran as a single
substance.

\[
\alpha^D_{\text{D}} = +60^\circ \text{ (equilibrium value, } H_2O, c = 2.0) \\
c.f. 2:6-di-0-methyl-D-glucose \quad \alpha^D_{\text{D}} = +63^\circ \text{ (eq.)} \\
(H_2O, c = 2.7) \quad (118)
\]
& 3:6-di-0-methyl-D-glucose \quad \alpha^D_{\text{D}} = +61^\circ \text{ (eq.)} \\
(H_2O, c = 3.3) \quad (119)

**Estimation of the amount of 2:6-di-0-methyl-D-glucose present in fraction 6 (120)**

A portion of fraction 6 (36 mg.) was heated
under reflux with methanolic hydrogen chloride solu-
tion (5 c.c.; 2%) for three hours, neutralised and
sodium metaperiodate (2 c.c.; 0.2M) added to the syrup obtained. After shaking in the dark at room temperature for five hours, phosphate buffer (2 c.c.; 0.5M; pH 7.5) and excess potassium iodide (2 c.c.; 20% aq.) were added to the solution and the amount of periodate consumed determined by titration with sodium arsenite (0.0403N).

It was found that the uptake of sodium metaperiodate was 0.83 moles per methyl di-2-methyl-D-glucoside unit. This indicated the presence of 83% 2:6-di-2-methyl-D-glucose in the fraction.

After estimation of the periodate uptake, ethylene glycol (1 c.c.) was added to the solution to destroy any excess periodate. The solution was then evaporated to dryness and the residue extracted several times with chloroform. The chloroform extracts were evaporated to dryness and the residue hydrolysed with sulphuric acid (2 c.c.; 2N) for 6 hours at 100° (bath temp.). After neutralisation the solution was examined by paper chromatography. One spot was seen corresponding to 3:6-di-2-methyl-D-glucose.

**Fraction 7**

On paper chromatograms using solvent 2 this fraction ran at the same speed as fraction 6 and
appeared to run as a single substance. Using solvent 1 the major portion of fraction 7 again ran at the same speed as fraction 6 but it was noticed that a third sugar was present which ran slower than the main fraction and gave a distinct yellow coloured spot when developed with aniline oxalate.

Separation of the sugars present in fraction 7 on a cellulose column.

A column of powdered cellulose was prepared as before and washed with 1 litre of water and then the solvent (solvent 1) to be used as eluant. Fraction 7 (50 mg.) was dissolved in the minimum amount of solvent and pipetted on to the top of the column. The constant level reservoir was inverted and the eluate collected in 5 c.c. fractions. The sugars did not separate very well and were washed through very quickly, but a separation into two portions was achieved.

Tubes 21 to 24 (incl.) contained only the suspected 2:6-/3:6-di-O-methyl-D-glucose mixture, and tubes 25 to 31 (incl.) this mixture plus the third sugar. The syrup left after the removal of the solvent from these latter tubes was spotted on to Whatman 3MM chromatography paper and the paper irrigated for 96 hours using solvent 1. After this time the paper was air dried, the side strips cut
off and sprayed with aniline oxalate, the appropriate parts of the main paper cut out and eluted with methanol. By this means a very good separation of the fraction into its two components was achieved.

Yield: - 40 mg. - suspected 2:6-/3:6- mixture Fraction 7A.

4 mg. - third sugar. Fraction 7B

44 mg.

Fraction 7A

This fraction gave a single spot on paper chromatograms and ran at the same speed as an authentic mixture of 2:6- and 3:6-di-0-methyl-D-glucose. Its methoxyl content was 29.5%. Demethylation gave only glucose.

\[
\frac{[\alpha]}{D} = +59^\circ \text{ (eq. value. } H_2O, \ c = 0.8)\
\]

Estimation of the amount of 2:6-di-0-methyl-D-glucose present in fraction 7A

Fraction 7A was dissolved in methanolic hydrogen chloride (5 c.c; 2%) and heated under reflux for three hours. The solution was then neutralised and treated with sodium metaperiodate as previously described.

It was found that the uptake of sodium metaperiodate was 0.80 moles per dimethyl methylglucoside unit indicating the presence of 80% of 2:6-di-0-methyl-D-glucose in the fraction.
The excess periodate was destroyed by addition of ethylene glycol and after working up the solution as previously described chromatographic investigation of the residue revealed the presence of a single sugar corresponding to 3:6-di-O-methyl-D-glucose.

**Fraction 7B**

This sugar had an $R_G$ value of 0.50 and gave a distinct yellow spot on development with aniline oxalate. Demethylation gave only glucose.

**Fraction 8**

Chromatographic examination of fraction 8 using solvent 2 revealed the presence of a single substance having an $R_G$ value of 0.20 - 0.22. Examination in solvent 1 however revealed the presence of three distinct substances, in the ratio of 1:1:1, one of which was fucose.

Fraction 8 (165 mg.) was spotted on to two Whatman 3MM papers and irrigated for 48 hours with solvent 1. Two main fractions were seen on developing the side strips with aniline oxalate - fucose and a mixture of the other two sugars present. The separation of the fucose from the faster moving sugars was not distinct so that part of each paper containing the sugars was divided into three - fucose only, fucose plus the faster moving sugars and faster moving sugars only. This latter part of each paper
was eluted with boiling water and the syrup so obtained examined. It was found that there was still some fucose present. Correspondingly the syrup was spotted on to a further Whatman 3MM paper and irrigated again for 48 hours using solvent 1. The paper was divided into three as previously and that part containing the faster moving sugars eluted as before. This time the separation from fucose was complete.

Yield: - 64 mg. Fraction 8B.

Demethylation gave only glucose. The methoxyl content was 16% (c.f. OMe = 16% calculated for a monomethyl hexose.)

Attempted separations of this fraction into its two components by use of paper chromatography proved unsuccessful.

**Characterisation of the monomethyl glucoses present in fraction 8B by periodate oxidation followed by alkaline hydrolysis (121)**

A portion of fraction 8B (1 mg.) was dissolved at 0° in sodium metaperiodate (0.120 c.c.; 0.5N) solution. After one hour at 0° ethylene glycol (2-3 mg.) was added to the solution and the mixture allowed to warm to room temperature. After 5 minutes the solution was made alkaline to phenolphthalein with sodium hydroxide solution (0.5N), then
<table>
<thead>
<tr>
<th>Saponified Periodate Oxidation Products From Pure Monomethyl Derivatives</th>
<th>Fraction 8</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-O-methyl-D-glucose</td>
<td>3-O-methyl-D-glucose</td>
</tr>
<tr>
<td>a. Lemon yellow</td>
<td>c. 2-O-methyl arabinose, plum</td>
</tr>
<tr>
<td>b. Faint canary yellow</td>
<td></td>
</tr>
<tr>
<td>R_f = 0.22.</td>
<td>R_f = 0.35.</td>
</tr>
<tr>
<td>FRACTION 8</td>
<td></td>
</tr>
<tr>
<td>p. Lemon yellow</td>
<td>q. Plum</td>
</tr>
<tr>
<td>R_f = 0.25.</td>
<td>R_f = 0.35.</td>
</tr>
</tbody>
</table>
after a further 5 minutes the solution was applied to paper chromatograms and these irrigated with solvent 2. The papers were dried and sprayed with a saturated solution of aniline phthalate. (122)

Figure 11 shows the results obtained compared with those obtained from authentic mono-O-methyl-D-glucose samples.

Examination of fraction 9

This fraction had a methoxyl content of 22.0%.

Lithium aluminium hydride reduction

A portion of the material (400 mg.) was dissolved in methanolic hydrogen chloride (20 c.c.; 2%) and heated under reflux for 10 hours. The solution was neutralised and the filtrate taken to dryness. Purified dioxan (10 c.c.) was added to the residue and the solution added to a well stirred suspension of lithium aluminium hydride (400 mg.) in purified dioxan (25 c.c.). The solution was stirred (mercury seal stirrer) under reflux for eight hours at 48° and then allowed to cool overnight before water was added to destroy the complex.

The solution was then acidified with 2N sulphuric acid, filtered, reduced in volume and extracted with chloroform in an all glass liquid/liquid
extractor.

The chloroform solution was taken to dryness and the residue hydrolysed with dilute hydrochloric acid. Examination of the products by paper chromatography revealed the following sugars present:

Table 8

<table>
<thead>
<tr>
<th>R&lt;sub&gt;0&lt;/sub&gt; value</th>
<th>Colour of spot</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.54</td>
<td>Brown</td>
</tr>
<tr>
<td>0.62</td>
<td>Red</td>
</tr>
<tr>
<td>0.75</td>
<td>Green</td>
</tr>
<tr>
<td>0.83</td>
<td>Red</td>
</tr>
</tbody>
</table>

The aqueous layer was taken to dryness, dissolved in methanol and de-ionised with Amberlite IR-100(H) ion exchange resin. After hydrolysis with dilute hydrochloric acid the residue was examined chromatographically and found to contain the following sugars:

Table 9

<table>
<thead>
<tr>
<th>R&lt;sub&gt;C&lt;/sub&gt; value</th>
<th>Colour of spot</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.04</td>
<td>Brown</td>
</tr>
<tr>
<td>0.11</td>
<td>Yellow</td>
</tr>
<tr>
<td>0.29</td>
<td>Brown</td>
</tr>
<tr>
<td>0.54</td>
<td>Brown</td>
</tr>
<tr>
<td>0.61</td>
<td>Red</td>
</tr>
<tr>
<td>0.79</td>
<td>Green</td>
</tr>
<tr>
<td>0.86</td>
<td>Red</td>
</tr>
</tbody>
</table>
Bromine oxidation followed by lithium aluminium hydride reduction

A further sample of the material from the water wash of the column (50.55 mg.) was dissolved in water and de-ionised on a column of Amberlite IR-120(H) ion exchange resin.

Yield 33.50 mg.  %Ba 33.7

This was dissolved in water (4 c.c.) and bromine (0.5 c.c.) added.  After 10 days at approximately 40° when the solution was non-reducing to Fehlings solution, the solution was filtered and neutralised with silver carbonate.  The syrup obtained was dissolved in methanolic hydrogen chloride (7 c.c.; 2%) and heated under reflux for 7 hours, then neutralised with silver carbonate.

The ester/glycoside obtained was dissolved in purified dioxan (1 c.c.) and lithium aluminium hydride (20 mg.) in dioxan (1 c.c.) added.  After standing at room temperature for 3 hours the complex was destroyed by the addition of water, the solution acidified with 2N sulphuric acid and cooled to 0°.  Barium hydroxide was added until the solution was alkaline, the solution centrifuged and the centrifugate tested with barium hydroxide to ensure that precipitation was complete. "Drikold" was then added until the precipitation of the carbonates was
complete, the solution filtered and taken to dryness. The residue was then extracted with methanol.

The extract was hydrolysed with hydrochloric acid (0.5N; 4 c.c.) for 7 hours and worked up as usual. Examination of the hydrolysis products by paper chromatography showed the following sugars to be present:

Table 10

<table>
<thead>
<tr>
<th>Rg value</th>
<th>Colour of spot</th>
<th>Opposite Standard</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.08</td>
<td>Brown</td>
<td>Glucose</td>
</tr>
<tr>
<td>0.24</td>
<td>Brown</td>
<td>Fucose</td>
</tr>
<tr>
<td>0.40</td>
<td>Pink</td>
<td></td>
</tr>
<tr>
<td>0.54</td>
<td>Brown</td>
<td>2:3-di-O-methyl-D-glucose</td>
</tr>
<tr>
<td>0.78</td>
<td>Green</td>
<td>3:5-di-O-methyl-L-fucose</td>
</tr>
<tr>
<td>0.84</td>
<td>Red</td>
<td>2:3:6-tri-O-methyl-D-glucose</td>
</tr>
</tbody>
</table>

Equivalent of fraction 9

A sample of the material (55.43 mg.) was dissolved in water and de-ionised as before.

Yield 35.72 mg. %Ba 33.6.

This weight was dissolved in sodium hydroxide (15 c.c.; 0.01054N) and allowed to stand at room temperature for one hour with occasional shaking. The excess alkali was determined by titration with hydrochloric acid (0.00982N).
The equivalent as found was 223.

Hydrolysis of fraction 9

A portion was hydrolysed with 2N sulphuric acid and examined by paper chromatography using as irrigant a solution of n-butanol, saturated with water to which 5% of 98% formic acid had been added. The following results were obtained:

<table>
<thead>
<tr>
<th>Colour of spot</th>
<th>Distance from starting line</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brown</td>
<td>2.5 cm.</td>
</tr>
<tr>
<td>Red</td>
<td>5 - 8 cm.</td>
</tr>
<tr>
<td>Cherry red</td>
<td>13.5 cm.</td>
</tr>
<tr>
<td>Cherry red</td>
<td>18.0 cm.</td>
</tr>
<tr>
<td>Brown</td>
<td>22.5 cm.</td>
</tr>
<tr>
<td>Cherry red</td>
<td>26 cm.</td>
</tr>
</tbody>
</table>

2:3-di-β-methyl-D-glucose run as standard moved 18.5 cm. A paper run in solvent 2 showed a large amount of streaking at the start and a brown spot at 20.7 cm. (2:3-di-β-methyl-D-glucose ran as standard travelled a distance of 18.5 cm.).

Hydrolysis of a further sample with 90% formic acid gave similar results except that a sugar giving a very definite spot at $R_g 0.80$ was present.

Attempted separation of fraction 9 on a column of ion-exchange resin (123)

Amberlite IRA-400(OH) ion exchange resin was thoroughly dried in vacuo over phosphorus pentoxide.
It was then ground down in a ball-mill for 6 hours, the resulting powder being further dried over phosphorus pentoxide until no moisture was present. The dry powder was mechanically sieved and all the material passing through a 100 mesh sieve was retained. This was suspended in water and the material settling between 10 and 30 minutes collected, then treated with 25% acetic acid until the evolution of carbon dioxide ceased.

A column of this material (27 x 1.5 cm.) was packed but the eluate came through too slowly, so the material was further fractionated and those particles settling between 10 and 20 minutes collected. A column (27 x 1.5 cm.) was packed with this material and found to run satisfactorily. The column was washed with 25% acetic acid (250 c.c.) then with water until the pH of the eluate was 7.0.

The remainder of fraction 9 (683.1 mg.) was deionised (Amberlite IR-100(H)) and the material obtained (420.2 mg. %Ba = 34.1) dissolved in the minimum amount of water and pipetted on to the column.

The eluate was collected in small test tubes changed at specific intervals by an automatic fraction collector and the solvent was contained in a constant head reservoir.
Water (1000 c.c.) was run through the column.

Yield of material 34 mg. (W.W.I).

Table 11 shows the solvents run through the column and the yields of material obtained.

In all cases the solvent was removed under reduced pressure at 30°, and after the concentration of acid reached 2.5% water was added periodically to the flask during solvent removal to lessen the concentration of acid.
Table 11

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Volume</th>
<th>Yield</th>
<th>Fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1% acetic acid</td>
<td>900 c.c.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.2%</td>
<td>1000 c.c.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.3%</td>
<td>1250 c.c.</td>
<td>10 mg.</td>
<td>W.W.2.</td>
</tr>
<tr>
<td>0.4%</td>
<td>1000 c.c.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5%</td>
<td>1000 c.c.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.6%</td>
<td>1000 c.c.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.7%</td>
<td>2500 c.c.</td>
<td>13 mg.</td>
<td>W.W.3.</td>
</tr>
<tr>
<td>0.8%</td>
<td>1000 c.c.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.9%</td>
<td>1000 c.c.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.0%</td>
<td>1000 c.c.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.2%</td>
<td>1000 c.c.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.4%</td>
<td>1000 c.c.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.6%</td>
<td>1000 c.c.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.8%</td>
<td>1500 c.c.</td>
<td>9 mg.</td>
<td>W.W.4.</td>
</tr>
<tr>
<td>2.0%</td>
<td>1000 c.c.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.5%</td>
<td>2000 c.c.</td>
<td>40 mg.</td>
<td>W.W.5.</td>
</tr>
<tr>
<td>3.0%</td>
<td>1000 c.c.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5.0%</td>
<td>1000 c.c.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7.5%</td>
<td>1000 c.c.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10.0%</td>
<td>5000 c.c.</td>
<td>104 mg.</td>
<td>W.W.6.</td>
</tr>
<tr>
<td>20.0%</td>
<td>2000 c.c.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>30.0% formic acid</td>
<td>3000 c.c.</td>
<td>150 mg.</td>
<td>W.W.7.</td>
</tr>
</tbody>
</table>

Total volume solvent = 32,150 c.c.
Material recovered from column = 360 mg.
Examination of the fractions

Fraction W.W.1

Examination by paper chromatography showed this fraction to be glucose, with accompanying traces of material held on the starting line in both neutral and acidic solvents.

Fraction W.W.2

This was a mixture of various substances as shown in the following table:

<table>
<thead>
<tr>
<th>R&lt;sub&gt;Gal&lt;/sub&gt;</th>
<th>Colour of spot</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>Pink</td>
</tr>
<tr>
<td>2.0</td>
<td>Pink</td>
</tr>
<tr>
<td>2.5</td>
<td>Red</td>
</tr>
<tr>
<td>3.0</td>
<td>Pink</td>
</tr>
<tr>
<td>3.3</td>
<td>Brown</td>
</tr>
<tr>
<td>3.8</td>
<td>Pink</td>
</tr>
<tr>
<td>5.0</td>
<td>Pink</td>
</tr>
</tbody>
</table>

Fraction W.W.3

This was composed mainly of one substance which gave a salmon pink spot at R<sub>Gal</sub> 3.8 but traces of contaminants were present.

Fraction W.W.4

This was again a mixture of substances.

Fraction W.W.5

Examination by paper chromatography using
solvents 2, 4 and 6 showed that this fraction ran at the same speed as a mixture of authentic 2:3:6-tri-O-methyl-D-glucose and 2:4:6-tri-O-methyl-D-glucose.

Rotation of W.W.5 in cold methanolic hydrogen chloride solution

W.W.5 was dissolved in methanolic hydrogen chloride (10 c.c.; 1%) to give a brilliant, golden yellow solution. Over a period of 24 hours the rotation fell from $+38^\circ$ to $+25^\circ$.

Fraction W.W.6

This had a methoxyl content of 18.9%. Examination in solvent 1 showed glucose and many other substances to be present.

Fraction W.W.7

This was a white solid with traces of brown syrup. Hydrolysis with 2N sulphuric acid gave glucose in very small yield. Examination by paper chromatography was inconclusive. Despite heavy spotting only faint traces of material were present when the paper was developed.

The solid slowly charred without melting above $290^\circ$. 
DISCUSSION
Very few attempts have been made to investigate the slime produced by micro-organisms of the Aerobacter-Klebsiella group although the fact that these organisms produce very mucoid growths has been known for some time.

Edwards (124) showed that these bacteria exist in a large number of immunologically distinct types and Wilkinson (loc. cit.) showed that these extracellular polysaccharides were responsible for the antigenic specificity of the strains. From the few investigations of Aerobacter polysaccharides which have been carried out very little information has been gathered about the chemical nature of the polysaccharides. However Wilkinson has started a systematic investigation of the extracellular polysaccharides produced by various strains of A. aerogenes and it is one of these which forms the subject of this thesis.

The polysaccharide was liberated into the medium by the organism as a viscous slime, and after isolation and purification was freeze-dried to give a pure white, spongy solid. The physical nature of the polysaccharide depended upon the method used for its final isolation. Thus by precipitating it from solution instead of freeze-drying the solution it
could be obtained as a grey fibre.

Wilkinson, Dudman and Aspinall (loc. cit.) grew the organism on media containing different carbon sources and identified and estimated the various constituent sugars formed on hydrolysis of the extracellular polysaccharide. They found that the composition of the polysaccharide did not vary with the carbon source. The object of this research was to investigate the polysaccharide further from a chemical point of view and to try to gain a greater insight into its structure.

Ultracentrifuge studies on the polysaccharide, carried out by Dr. C. T. Greenwood, indicated that the polysaccharide was homogeneous and that there were no contaminating substances present, but in the absence of further data no estimation of its molecular weight can be made.

A notable feature of the polysaccharide was the extreme viscosity of its solutions even at high dilution. Concentrations of the order of 1% caused the solution to gel.

The first batch of polysaccharide had a methoxyl content of 3.8% but as this was removable by dilute alkaline hydrolysis it did not imply etherification of any groups. Further batches of polysaccharide
did not have this methoxyl content.

Standard methods of hydrolysis, 1N and 2N sulphuric acid, even for 24 hours proved very ineffective when applied to the polysaccharide and it became evident at an early stage in the investigation that the polysaccharide was extremely resistant to chemical methods of breakdown. Eventually it was found that a reasonable hydrolysis could be achieved by using 98% formic acid for 24 hours at 100°, followed by N-sulphuric acid for 6 hours and this was adopted as the standard method for quantitative studies although some degradation did occur. The sugars liberated were D-glucose, galactose and L-fucose.

The D-glucose was separated on chromatograms and identified by formation of crystalline 1:2:3:4:6-penta-O-acetyl-β-D glucopyranose. Fucose was similarly separated and identified by formation of crystalline L-fucose phenylhydrazone. Galactose, however, has only been shown to be present by paper chromatography. There was not enough material present to allow it to be identified by the formation of a crystalline derivative.

Dudman and Wilkinson have also isolated an extracellular O-polysaccharide from A. aerogenes which they have proved to be a galactan. They suggest that the
galactose present in the polysaccharide under investigation has no structural significance but is a contaminant from the extracellular \(\alpha\)-polysaccharide.

Estimation of the amount of each sugar present was complicated by the fact that it is very difficult to separate glucose and galactose from each other on paper chromatograms. A fairly sharp separation can be achieved by the use of a long irrigation time but the situation was rendered difficult by the presence of L-fucose which travels relatively quickly on chromatograms. Longer irrigation periods would have meant excessively long chromatograms which would probably have decreased the efficiency of the separation. A compromise had therefore to be reached with the result that the separation of D-glucose and D-galactose was not complete.

The amount of each sugar present was found to be:

- D-glucose 38.3\%; D-galactose 4.4\%; L-fucose 10\%.

Dudman's figures for the same material are:

- D-glucose 46\%; D-galactose 1-2\%; L-fucose 9-10\%.

The percentage uronic acid present was 25.8 from the equivalent, 28 as determined by decarboxylation and 25.5 by the method of Kaye and Kent, which involves reacting the carboxylic acid ester with hydrox-
ylamine and ferric chloride.

Thus the total amount of sugars accounted for lies between the limits 77-86%. Ash content (2%), nitrogen content (0.2%) and phosphorus content (0.1%) of the polysaccharide bring these limits up to approximately 80-88% leaving 16-20% of the weight of the polysaccharide unaccounted for. It is well known that polyuronides are difficult to hydrolyse and the conditions required for hydrolysis lead to the formation of furfural and liberation of carbon dioxide and undoubtedly this occurred in the rather drastic hydrolysis of the polysaccharide under investigation. Even with this hydrolysis however there still remained an unhydrolysed residue which liberated glucose, galactose and fucose on further hydrolysis. Thus the amounts of neutral sugars present may be greater than given above thus accounting for the discrepancy in the percentage composition of the polysaccharide.

The nature of the uronic acid present was not indicated by this hydrolysis but treatment of the polysaccharide with 4N hydrochloric acid liberated glucurone in small amount. This was only shown to be such by paper chromatography.

Graded hydrolysis of the polysaccharide with 0.5N acid liberated glucose after five minutes at
100° but thirty minutes hydrolysis with this strength of acid was found to give the optimum yield of products suspected of being di- and trisaccharides. The hydrolysate was separated into two acidic fractions (A & B) and a neutral fraction. The two acidic fractions appeared to be basically the same as far as could be ascertained from the nature of the products liberated from them on further hydrolysis.

The sugars in the neutral fraction were D-glucose, characterised as 1:2:3:4:6-penta-O-acetyl-β-D-glucopyranose and cellobiose, characterised as α-octa-acetyl cellobiose. Galactose was also suspected to be present together with a possible disaccharide which liberated glucose and galactose on further hydrolysis. A large part of the neutral fraction was composed of an unhydrolysed residue. The sugars liberated by hydrolysis of this residue appeared to be the same as those liberated by hydrolysis of acidic fraction A, so the two residues were combined and hydrolysed with 2N sulphuric acid.

From this hydrolysate ten fractions were separated. The sugars characterised were L-fucose, as L-fucose p-toluene sulphonhydrazone and D-glucose as 1:2:3:4:6-penta-O-acetyl-β-D-glucopyranose. Galactose was again suspected to be present but no
crystalline derivative could be formed.

Two disaccharides were suspected to be present, one travelled at the same speed as cellobiose on paper chromatograms the other slightly faster. The latter gave glucose and fucose on hydrolysis but an attempted formation of α-octa-acetyl cellobiose from the former was unsuccessful.

All the remaining five fractions were oligosaccharides. The fastest moving of these contained only neutral sugars but the remaining four are suspected to be acidic in character. This supposition is based on their behaviour on paper chromatograms in neutral and acidic solvents and on the liberation of glucurone from them by hydrolysis with 4N hydrochloric acid.

As only 20-30 mg. of each were isolated very little could be done to investigate their nature other than by noting their behaviour on paper chromatograms and by hydrolysis.

The neutral oligosaccharide gave galactose, glucose and fucose on hydrolysis with 2N sulphuric acid. The acidic oligosaccharides required 4N hydrochloric acid for their hydrolysis and this liberated glucose, fucose and glucurone. After treatment with methanolic hydrogen chloride the
acidic oligosaccharides were reduced with potassium borohydride and again hydrolysed with 4N hydrochloric acid. The sugars liberated on hydrolysis were glucose and fucose, and in three cases a trace of galactose.

It would seem therefore that the galactose residues are very strongly bound in the polysaccharide and this might account for the variable, small amount liberated on hydrolysis. The above evidence also suggests that the galactose residues are constituent units of the polysaccharide and do not come from a contaminating extracellular O-polysaccharide.

Apart from galactose, present in trace amount in three of the hydrolysates of the reduced oligosaccharides, no hexose residue, other than glucose, was liberated. After reduction of the oligosaccharides glucose was present in enriched amount in the hydrolysates. This evidence suggests very strongly that the uronic acid residue present in the polysaccharide is glucuronic acid.

Thus from the results obtained by graded hydrolysis of the polysaccharide it has been shown that the linkage:

......xGlu.(p)l - 4Glu.(p)x.... exists.

Glu.(p) = D-glucopyranose.
1 and 4 refer to the points of linkage and x represents an unknown point of linkage.

The ratio of glucose to fucose in the sugars liberated on hydrolysis of the polysaccharide is approximately 4/1. If the uronic acid present is glucuronic acid then after reduction of the polysaccharide and hydrolysis there should be a detectable increase in the amount of glucose present in the hydrolysate.

The first method tried was that of methanolysing the polysaccharide and then reducing it. The results of this experiment were:

\[ \text{Glucose/fucose } 5.6/1 \]

However this is open to criticism on the grounds that no check was kept on the amount of each sugar degraded and the increase in the ratio may have arisen from a decrease in the amount of fucose present. Furthermore it was felt that the conditions of hydrolysis were not drastic enough, taking into consideration the nature of the polysaccharide. Therefore in the second experiment two samples of the polysaccharide were subjected to a 24 hour hydrolysis with formic acid then L-rhamnose weighed in as a reference sugar. After sulphuric acid hydrolysis one sample was reduced, the other was left unreduced.
After a further hydrolysis the sugars present in both samples were separated on chromatograms and the amount of each sugar present estimated by the method of Somogyi. The results were:

Unreduced sample  Glucose/Fucose  4/1
Reduced sample    Glucose/Fucose  5.8/1

Since the ratio of rhamnose to fucose was constant, the increase in the ratio of glucose to fucose must be due to the reduction of D-glucuronic acid residues to glucose.

It was reasoned that reduction of the polysaccharide under investigation, followed by graded hydrolysis with dilute acid might liberate some disaccharides in which the glucose residue present was formerly a glucuronic acid residue. But the experiment designed to do this met with no success.

Methylation of the polysaccharide by sodium hydroxide and methyl sulphate proved extremely difficult and a very poor yield of methylated material was obtained. The methoxyl content of the fully methylated polysaccharide was 42%.

Preliminary small scale hydrolyses of this material with methanolic hydrogen chloride and formic acid had shown that the latter was the most effective hydrolysing agent and this was therefore chosen for
the large scale hydrolysis. But it was not appreciated from these experiments that the concentration (w/v) of the polysaccharide in the formic acid was an all important factor and consequently in the large scale hydrolysis too high a concentration of methylated polysaccharide in the formic acid was used with the result that considerable charring occurred. The hydrolysis products (28% yield) were separated into an acidic and a non-acidic fraction.

The attempted separation of the products of the above hydrolysis on a cellulose column met with little success and the investigation was stopped.

Since the Haworth methylation was not successful an attempt was made to methylate the polysaccharide with sodium and methyl iodide in liquid ammonia, but no methoxyl groups could be introduced at all, possibly due to the physical nature of the polysaccharide.

Although not very satisfactory the results achieved by using thallous hydroxide and methyl iodide to methylate the polysaccharide proved the best of those so far obtained. Here again however extreme difficulty was found in introducing methoxyl groupings and yields were low. Whereas other workers using this technique (103, 106) methylated their material
to a high degree after one or at the most two complete treatments, the polysaccharide under investigation required many methylations before the methoxyl value was even near the maximum. As the methoxyl value was raised the methylated material became increasingly insoluble in the benzene required as solvent during the methylation and yields thereby decreased very rapidly. No effective remedy was found to counteract this. In the first of the methylations acetone was tried as an alternative solvent to benzene, but on addition of thallous ethoxide the acetone polymerised. Although the solution was quickly extracted and the extracts subjected to a thorough purification treatment there was no doubt that an appreciable amount of contaminating material was not removed.

In the subsequent fractionation of the methylated material it became increasingly clear that only partial methylation of the polysaccharide had been achieved as from 3.5 g. of methylated material only 1.4 g. fully methylated (OMe = 42%) polysaccharide was obtained. This was hydrolysed with formic acid and separated into an acidic and a neutral portion by formation of the barium salt.

The neutral sugars were separated on a cellulose column and five main fractions obtained. It was soon
recognised however that these fractions were not individual sugars but mixtures of sugars with similar Rg values.

The tetra-O-methyl and tri-O-methyl fractions were further separated on a cellulose column using solvent 6 as eluant. Through an unfortunate occurrence however the sugars became contaminated with polymer from a "Polythene" tube used on the bottom of the column. Removal of this was effected by separating the three fractions recovered from the column on Whatman 3MM papers, but undoubtedly the sugars that were eluted from the papers were still slightly contaminated. Thus the weights of fractions X1, X2 and X3 are not the weights of the sugars they contain.

The sugar present in fractions X1 and X2 was proved to be 2:3:4:6-tetra-O-methyl-D-glucose by a mixed melting point with an authentic sample of the sugar. The small amount of this sugar present, less than 2% of the weight hydrolysed, indicates that only a small percentage of the glucose residues must be present as end-group.

Fraction X3 was shown to be a mixture of tri-O-methyl-D-glucoses but separation of these proved impossible. The evidence strongly suggests that the
sugars present were 2:4:6-tri-O-methyl-D-glucose and 2:3:6-tri-O-methyl-D-glucose. The presence of 2:3:6-tri-O-methyl-D-glucose was indicated more fully by the change in rotation of the fraction in cold methanolic hydrogen chloride. By this means it was estimated that this sugar was present to the extent of approximately 59%. Separation of these two isomers by paper chromatography is almost impossible since their $R_g$ values are so close together (0.83 and 0.85 respectively). A slight distinction can be noted from the colour of the spot each sugar gives when developed with aniline oxalate. Thus if the experimental conditions are carefully chosen 2:4:6-tri-O-methyl-D-glucose gives a red spot whilst 2:3:6-tri-O-methyl-D-glucose gives a brown spot, and a mixture of the two appears as one large spot, red on top and brown underneath.

Mixtures of these two sugars have been partially separated but only when large quantities have been available (125) (126) (127).

From observations noted on a small scale experiment it was thought that cold methanolic hydrogen chloride might preferentially attack the 2:3:6-isomer in a mixture of 2:3:6-tri-O-methyl and 2:4:6-tri-O-methyl-D-glucoses thereby affording a possible means
of separation but when attempted on a large scale this proved ineffective (129).

Fraction 2 was also a mixture. It contained a sugar which gave a very characteristic green colouration on development with aniline oxalate and traces of the sugars present in fraction X3. The main constituent was shown by demethylation experiments to be a derivative of fucose. At first it was thought that separation of fraction 2 into its components was impossible but this was later achieved by the use of a cellulose column and crystalline di-α-methyl-L-fucose was obtained. This was shown (see later) to be 3:5-di-α-methyl-6-deoxy-L-galactose and is the first reported isolation of this sugar.

The last three fractions were all mixtures of sugars which proved impossible to separate. Their components were therefore not identified.

The acidic fraction from this methylation proved equally difficult to investigate. In the first lithium aluminium hydride reduction dioxan was used as solvent since ether would not dissolve the syrup. The yields from each stage were very poor and only sufficient material was left at the completion of the experiment to examine it chromatographically. A substance was present which had an $R_G$ value of 0.58, and demethylation of it gave only glucose.
The second reduction using tetrahydrofuran as solvent and a modified isolation procedure gave much better yields than the first reduction but as equally inconclusive results. From the nature of the products isolated (R\text{G} 0.86; 0.57; 0.30; 0.21) it seems reasonable to postulate that the acidic fraction was in fact an unhydrolysed portion of the methylated polysaccharide containing both neutral and acidic sugar residues. The fact that it was an unhydrolysed fragment would explain the failure to form a lactone or amide.

Investigations on this material were stopped at this stage and begun anew on a fresh batch of polysaccharide.

This was methylated with thallous hydroxide and methyl iodide and hydrolysed with methanolic hydrogen chloride. The hydrolysis were separated on a cellulose column. Great care was taken during the elution of the column and a reasonable degree of success was obtained in the separation of the sugars.

The amount of 2:3:4:6-tetra-\text{O}-methyl-D-glucose present, characterised as its crystalline anilide, was very small, but this is consistent with previous findings.

The major tri-\text{O}-methyl fraction was the same.
mixture of tri-0-methyl-D-glucoses obtained before. From the behaviour of the fraction in cold methanolic hydrogen chloride the amount of 2:3:6-tri-0-methyl-D-glucose was estimated to be 36%.

More attempts were made to find a method which would separate these two isomers. Abdel-Akher and Smith (loc. cit.) have developed a spray which forms a blue or mauve colour with esters and lactones. In the present work it was found that this spray is not specific and small amounts of reducing sugars give reddish brown colours. Definition is better if the paper is allowed to remain at room temperature for 12 hours after spraying with the ferric chloride.

Abdel-Akher and Smith separated lactones and esters on paper chromatograms and identified them. Correspondingly an attempt was made to prepare 2:4:6-tri-0-methyl-D-glucono-1,5 lactone and 2:3:6-tri-0-methyl-D-glucono-1,4 lactone from a synthetic mixture of the corresponding tri-0-methyl-D-glucoses. The lactones were separated on paper chromatograms but due to low yields insufficient quantities were obtained to form crystalline derivatives.

Lindberg and Wickberg (loc. cit.) separated a mixture of 2:3:6-tri-0-methyl-D-glucose and 2:3:4-tri-0-methyl-D-glucose on a charcoal/celite column by
graded elution of the column and obtained a 96% recovery of 97% pure sugars. This method was tried with the synthetic mixture of tri-\(\beta\)-methyl-D-glucoses, but no separation was achieved.

Fraction 4 was shown to be a di-\(\beta\)-methyl-L-fucose and a search of the literature failed to reveal any known di-\(\beta\)-methyl-L-fucose with the same properties. Chromatograms were run with standards of 3:4-di-\(\alpha\)-methyl-L-fucose and 2:3-di-\(\alpha\)-methyl-L-fucose, but the unknown travelled much faster. \((R_\alpha 0.80\) as compared with \(R_\alpha 0.67\) and 0.65 for 3:4-di-\(\alpha\)-methyl-L-fucose and 2:3-di-\(\alpha\)-methyl-L-fucose respectively).

Charalambous (107) synthesised a di-\(\alpha\)-methyl-L-fucose which he proposed as the 2:4-di-\(\alpha\)-methyl derivative, but its properties differ markedly from those of the unknown.

Applying Hudson's lactone rule the positive rotation of the lactone indicated a \(\beta\)-lactone and this was substantiated by the slow rate of hydrolysis of the lactone. The rate of hydrolysis of the methyl glycoside indicated a furanose ring structure. Therefore the methoxyl groups were attached to carbon atoms 2 and 5 or 3 and 5.

Periodate oxidation showed the uptake of 1 mole/mole. The 3:5-di-\(\alpha\)-methyl derivative would take up
1 mole of periodate but the 2:5-di-O-methyl derivative would not react with periodate unless the ring opened and the molecule reacted in the open chain form.

Final proof of the true nature of the molecule was obtained from a study of the Weerman reaction on the amide of the di-O-methyl fuconic acid. This indicated that carbon atom 2 must possess a free hydroxyl group since hydrazodicarbonamide was formed. Thus the sugar can only be 3:5-di-O-methyl-6-deoxy-L-galactose. As mentioned previously it was eventually obtained as a crystalline substance of m. pt. 118-121°.

Fraction 5 was shown to be 2:3-di-O-methyl-D-glucose and 2-O-methyl-L-fucose was isolated from fraction 6. From the behaviour on chromatograms, demethylation studies and the behaviour in cold methanolic hydrogen chloride the rest of fraction 6 was suspected to be a mixture of 2:6-di-O-methyl-D-glucose and 3:6-di-O-methyl-D-glucose. This mixture is often encountered in studies on starch as a result of undermethylation and demethylation.

The constituent sugars of this fraction were not characterised by formation of crystalline derivatives but the amount of each component was estimated by periodate oxidation.

The methyl glycoside of 2:6-di-O-methyl-D-glucose
will undergo oxidation by the periodate ion by virtue of two adjacent hydroxyl groups on carbon atoms 3 and 4, but methyl 3:6-di-\(\text{O}\)-methyl-D-glucopyranoside has no two hydroxyl groups adjacent and cannot therefore undergo oxidation by the periodate ion. So measurement of the periodate uptake by a mixture of these two sugars will give an indication of the amount of each sugar present.

Thus in fraction 6 after removal of the 2-\(\text{O}\)-methyl-L-fucose 83.5% of the remainder was 2:6-di-\(\text{O}\)-methyl-D-glucose. Similarly 80% of fraction 7 was composed of 2:6-di-\(\text{O}\)-methyl-D-glucose. It is doubtful if all the weight of the di-\(\text{O}\)-methyl-D-glucoses comes from their presence as branch points in the molecule. Allowance must be made for some degree of undermethylation of the polysaccharide and some degree of demethylation does occur when methylated polysaccharides are subjected to methanolysis and hydrolysis. Undermethylation is almost unavoidable especially in a very complex polysaccharide like the one under investigation, but the amount of demethylation depends upon the concentration of the reagents used and the time of contact of these with the methylated sugars.

MacWilliam and Percival (129) have reported up
to 1% demethylation occurring when 2:3:6-tri-0-methyl-D-glucose was subjected to hydrolysis with 1% hydrochloric acid. Similar results were reported by Chanda who obtained di-0-methyl-D-glucose and mono-0-methyl-D-glucose by treatment of 2:3:6- and 2:4:6-tri-0-methyl-D-glucoses with N hydrochloric acid for 3 hours.

The last fraction of the neutral part of the molecule contained fucose along with two mono-0-methyl-D-glucoses. The indications were that these latter two sugars were 2-0-methyl-D-glucose and 3-0-methyl-D-glucose.

Fraction 9 was the "acidic" portion of the methylated polysaccharide as its barium salt. As in the previous methylation this proved to be an unhydrolysed residue. Reduction of the uronic acid present with lithium aluminium hydride allowed the fraction to be readily hydrolysed to give a complex mixture of methylated and unmethylated sugars. From the nature of these it would appear that the size of this unhydrolysed fraction was greater than the one isolated from the previous methylation probably because the hydrolysis with methanolic hydrogen chloride was less drastic than the treatment with formic acid.
An attempt was made to separate the acidic fraction into its components by graded elution from a column of ion exchange resin but this met with no success. The only sugars recovered from the column were D-glucose and what was suspected to be a mixture of 2:3:6- and 2:4:6-tri-0-methyl-D-glucoses. These latter sugars probably came from a larger fragment which was broken down when the acidic eluate was taken to dryness.

If it is valid to calculate the molar ratios of the sugars present in the neutral part of the molecule then the following results are obtained:

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Wt. (mg.)</th>
<th>% by wt.</th>
<th>M. Mol. proprn.</th>
<th>Mol. %</th>
<th>Mol. ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>8</td>
<td>1.1</td>
<td>237 0.005</td>
<td>0.96</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>5</td>
<td>0.7</td>
<td>223 0.003</td>
<td>0.64</td>
<td>0.7</td>
</tr>
<tr>
<td>3</td>
<td>122</td>
<td>17.3</td>
<td>223 0.077</td>
<td>15.5</td>
<td>16</td>
</tr>
<tr>
<td>4</td>
<td>77</td>
<td>10.9</td>
<td>192 0.057</td>
<td>11.4</td>
<td>12</td>
</tr>
<tr>
<td>5</td>
<td>45</td>
<td>6.4</td>
<td>209 0.030</td>
<td>6.1</td>
<td>6</td>
</tr>
<tr>
<td>6(Glu.)</td>
<td>121</td>
<td>17.1</td>
<td>209 0.082</td>
<td>16.4</td>
<td>17</td>
</tr>
<tr>
<td>6(Fuc.)</td>
<td>43</td>
<td>6.1</td>
<td>178 0.034</td>
<td>6.8</td>
<td>7</td>
</tr>
<tr>
<td>7</td>
<td>97</td>
<td>13.7</td>
<td>209 0.065</td>
<td>13.0</td>
<td>14</td>
</tr>
<tr>
<td>8</td>
<td>126</td>
<td>17.8</td>
<td>194 0.092</td>
<td>18.4</td>
<td>19</td>
</tr>
<tr>
<td>Fucose</td>
<td>63</td>
<td>8.9</td>
<td>164 0.054</td>
<td>10.9</td>
<td>11</td>
</tr>
</tbody>
</table>

It is obvious that the polysaccharide is a very
complex substance. Periodate oxidation showed that 1 mole of formic acid was produced per 2-3 anhydroglucose units and the uptake of periodate was 1.3 moles per anhydroglucose unit. After the uptake was constant hydrolysis of the degraded polysaccharide liberated D-glucose and L-fucose the former sugar in quantity. There were also traces of D-galactose present. These sugars could only have come from those residues in the original polysaccharide which were not open to attack by periodate. Therefore they must have possessed no contiguous hydroxyl groupings.

Thus the isolation of 2-O-methyl-D-glucose, 3-O-methyl-D-glucose, 2:6-di-O-methyl-D-glucose, 3:6-di-O-methyl-D-glucose, 2:4:6-tri-O-methyl-D-glucose, 2-O-methyl-L-fucose, and 3:5-di-O-methyl-L-fucose from the products of hydrolysis of the methylated polysaccharide can be attributed to this, bearing in mind the possibilities of demethylation and undermethylation mentioned previously. It is interesting to speculate on the isolation of free glucose and free fucose from the products of hydrolysis of the methylated polysaccharide. This is possibly due to their acting as multiple branch points or to demethylation or undermethylation.

The traces of galactose found on hydrolysis of
the oxidised polysaccharide would seem to indicate that this sugar too acted as a branch point, or was linked 1,3 but no methylated derivatives of this sugar were found.

The large amount of mono-O-methyl and di-O-methyl sugar isolated and the very small amount of tetra-O-methyl end group (66 parts to 1 part) lead to two postulations.

The molecule could be a branched chain structure in which case a large amount of end-group is unaccounted for. The ratio of mono-O-methyl and di-O-methyl sugar to end-group must be 1:1 in this case. The only possibility left is that this end group is present in the unhydrolysed part of the molecule. It is unlikely that this end group is 2:3:4:6-tetra-O-methyl-D-glucose because in all the investigations of the unhydrolysed part of the methylated polysaccharide no suggestion of this sugar was ever found. The other possibility is that some of the uronic acid residues form the end group probably as the tri-O-methyl derivative.

If the polysaccharide is not a branched chain then it is possible that the molecule has a "net-like" structure in which each chain is linked to the next by branch chains. In this case the amount of mono-
O-methyl and di-O-methyl sugars would be very much greater than the amount of end group.

At this stage however it is impossible to put forward a unique molecular structure for the polysaccharide.

The sugar residues whose identity is established are:

- Glu.(p)l....; ....2Fuc.(f)l....; Glu.(p)l....;
- Fuc.(p)l....; ....4Glu.(p)l....; ....3Glu.(p)l....;

and those whose identity is strongly indicated are:

- Glu.(p)l....; Glu.(p)l....; 4Glu.(p)l....;
- 3Glu.(p)l....;
- 4Glu.(p)l....;

The linkage ....xGlu.(p);-4Glu.(p)x.... also exists.

Glu.(p) - D-glucopyranose
Fuc.(p) - L-fucopyranose
Fuc.(f) - L-fucofuranose

The numbers refer to the points of linkage and x is an unknown point of linkage.

Until a method of fully hydrolysing the methylated and unmethylated polysaccharide is found and di- and tri-saccharides are isolated and studied then the more detailed structure of the polysaccharide must remain in doubt.
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