Structures of Polysaccharides Elaborated by Cariogenic Organisms

by

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A thesis presented to the Faculty of Science of the University of London in candidature for the degree of Doctor of Philosophy

Royal Holloway College (University of London) Egham Hill Egham Surrey

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789 East Eisenhower Parkway
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Ann Arbor, MI 48106-1346
To my wife and my parents
ACKNOWLEDGEMENTS

The author wishes to thank Royal Holloway College for financial assistance;
The late Professor E.J. Bourne for the use of the laboratories and facilities and
Dr. H. Weigel for his excellent guidance and assistance throughout the work.
The author also wishes to thank Dr. W.H. Bowen and Dr. E.E. Percival for their gifts of bacteria and g.l.c. standards respectively, Mr. B. Smothurst and Mr. A.S. Ashdown for their technical assistance in the modification of the g.l.c. – m.s. system and Dr. R.L. Sidebotham for occasional discussions and much valuable information concerning the Chemistry of Dextrins.
The structures of the extracellular polysaccharides elaborated by Streptococcus mutans GS-5 and S. mutans OMZ 51 were investigated.

These were fractionated into soluble fractions (GS-5B and OMZ 51B polysaccharides) and lesser-soluble fractions (GS-5A and OMZ 51A polysaccharides).

GS-5B polysaccharide was essentially an α-(1→6)-linked polyglucan in which 17% of the glucose units were linked through positions 1, 3 and 6 and formed branch points. 60% of the molecule comprised a single α-(1→6)-linked glucose chain and the minimum average external chain length was 2. The polysaccharide was poly-dispersed $\bar{M}_w = 49,000$ with a structurally similar low molecular weight fraction $M_w / M_n = 10,000$. The following average repeating unit was proposed.

\[
\begin{align*}
1 & \quad 6 \\
D\text{-glicp} & \xrightarrow{1} D\text{-glicp}
\end{align*}
\]

GS-5A polysaccharide contained approximately 35% of fructose and 40% of glucose. The polyglucan was predominately α-(1→6)-linked and 20% of the glucose units were linked through positions 1, 3 and 6 and formed branch points. Approximately 3% of the glucose units were linked through positions 1 and 3 only.

An average repeating unit was proposed.
OMZ 51B polysaccharide was essentially an α-(1→6)-linked polyglucan in which 14% of the glucose units were linked through positions 1, 3 and 6 and formed branch points. The polysaccharide was polydispersed $M_w = 32,000$ and the minimum average external chain length was 2. The following average repeating unit was proposed.

OMZ 51 A polysaccharide was predominantly an α-(1→6)-linked polyglucan in which 20% of the glucose units were linked through positions 1, 3 and 6 and formed branch points and 3.7% were linked through positions 1 and 3 only. Enzymic degradation studies indicated that some of the latter occurred at non-branch points within α-(1→6)-linked glucose chains. The minimum average external chain length was 1.6. An average repeating unit was proposed.

Several polysaccharides elaborated by carcinogenic microorganisms and several bacterial dextrans were treated with swine kidney glucanhydrolase. Conversions to glucose of 1 - 15.5% were observed.
## CHAPTER I

### INTRODUCTION

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>IA  A Brief Review of the Chemistry of Dextran</td>
<td>1</td>
</tr>
<tr>
<td>IA 1 Origin, Production and Uses</td>
<td>1</td>
</tr>
<tr>
<td>IA 2 Types and Percentages of Various Glucosidic Linkages in Dextran</td>
<td>3</td>
</tr>
<tr>
<td>IA 3 Structural Segments obtained from Dextran</td>
<td>10</td>
</tr>
<tr>
<td>IA 4 The Lengths of Branches in Dextran</td>
<td>20</td>
</tr>
<tr>
<td>IA 5 The Biosynthesis of Dextran</td>
<td>27</td>
</tr>
<tr>
<td>IA 6 Conclusions</td>
<td>33</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>IB  Dextran and Dental Caries</td>
<td>35</td>
</tr>
<tr>
<td>IB 1 The Basic Requirements for the Production of Dental Caries</td>
<td>35</td>
</tr>
<tr>
<td>IB 2 The Development of the Dental Plaque and the Colonisation of the Tooth Surface by Oral Bacteria</td>
<td>42</td>
</tr>
<tr>
<td>IB 3 Caries Prevention and the Control of Dental Plaque</td>
<td>46</td>
</tr>
<tr>
<td>IB 4 The Biochemistry of the Dental Plaque</td>
<td>50</td>
</tr>
</tbody>
</table>

## CHAPTER II

### THE POLYSACCHARIDE ELABORATED BY STREPTOCOGGUS MUTANS GS-5

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ITA  Preparation and Extraction</td>
<td>57</td>
</tr>
<tr>
<td>ITB  The GS-5 P polysaccharide</td>
<td>58</td>
</tr>
<tr>
<td>ITB 1 Monosaccharide components by acid hydrolysis</td>
<td>58</td>
</tr>
<tr>
<td>ITB 2 Composition</td>
<td>59</td>
</tr>
<tr>
<td>ITB 3 The ratio of primary to secondary linkages by n.m.r. spectroscopy</td>
<td>60</td>
</tr>
<tr>
<td>ITB 4 The infrared spectrum</td>
<td>62</td>
</tr>
<tr>
<td>ITB 5 Percentages of variously linked D-glucose residues by methylation analysis</td>
<td>63</td>
</tr>
<tr>
<td>ITB 6 Characterisation of primary linkages by partial acid hydrolysis</td>
<td>80</td>
</tr>
<tr>
<td>ITB 7 Characterisation of secondary linkages by partial acetylolation</td>
<td>87</td>
</tr>
</tbody>
</table>
IIB 8  Enzymic degradation by *P. lilacinum* dextranase 99
IIB 9  Molecular weight distribution by gel permeation chromatography 107
IIB 10 Polysaccharide structure in the region of the branch points by mild acid hydrolysis 117
IIB 11 Minimum lengths of external chains by swine kidney glucanhydrolase hydrolysis 124

IIC  The GS-5 A Polysaccharide 135
IIC 1 Monosaccharide components by acid hydrolysis 135
IIC 2 Composition 136
IIC 3 Percentages of variously linked α-glucose units by methylation analysis 137
IIC 4 Enzymic degradation by *P. lilacinum* dextranase 141
IIC 5 Minimum lengths of external chains by swine kidney glucanhydrolase hydrolysis 142

IID  Conclusions

CHAPTER III  THE POLYSACCHARIDE ELABORATED BY STREPTOCOCCUS MUTANS ONZ 51
III A  Preparation and Extraction 149

III B  The ONZ 51 B Polysaccharide 150
III B 1 Monosaccharide components by acid hydrolysis 150
III B 2 Composition 150
III B 3 Percentages of variously linked α-glucose units by methylation analysis 151
III B 4 Enzymic degradation by *P. lilacinum* dextranase 152
III B 5 Minimum lengths of external chains by swine kidney glucanhydrolase hydrolysis 153
III B 6 Molecular weight distribution by gel permeation chromatography 155
Chapter I

INTRODUCTION

1A A brief Review of the Chemistry of Dextran

1A1 Origin, production and uses

Dextran are β-glucans in which a substantial percentage of the β-glucopyranosyl units are α-(1 → 6)-linked. They are produced when certain bacteria derived from the genera Leuconostoc1-3, Lactobacillus4-10 and Streptococcus1,4,11-21, are grown on a sucrose substrate, although dextrans have been synthesised by bacteria growing on other substrates22,23 and the synthesis, by chemical means of an essentially unbranched dextran has been reported24,25.

The dextrans are extracted from cell-free cultures of micro-organisms1,17,18,26-35 and are purified by deproteinisation and repeated alcohol or ketone precipitation1,26,27,29,36,37.

Difficulties are encountered in the purification of the polysaccharides elaborated by Streptococci, since these organisms tend to produce extracellular fructans in addition to the dextrans17,18,38,39 although a partial separation can sometimes be obtained by ethanol precipitation27,40.

There are three major areas in which the study of dextran is of particular interest. Dextran have been implicated in the caries process (see section 1B), they are produced as a by-product in the sugar refining industry41-45, and they are used as blood plasma expanders in medical practice46-49.

The synthesis of dextran by soil micro-organisms, principally Leuconostoc, from sugar cane has been a major difficulty in the sugar industry for some time. These by-products have the effect of directly reducing the yield of sucrose, increasing sucrose syrup viscosities50-55.
and adversely effecting the crystallisation of sucrose $^{51,56,57}$.

In the area of sugar cane production this problem has been intensified with the advent of mechanisation $^{58}$. When the sugar cane was cut by hand, harvesting produced a single cut surface per six foot length of cane. The Leuconostoc micro-organisms metabolised the sucrose substrate at the cut surface producing dextran, but the dextran was produced in quantities too small to have a great effect upon the milling and refining processes.

When harvesting was mechanised, chopper-harvesters cut the canes into approximately nine inch lengths, so producing an additional fifteen cut surfaces per cane. Dextran elaboration by the Leuconostoc then became significant. The effect of the dextran was to retard the rate of growth on certain faces of the sucrose crystal, causing $c$-axis elongation $^{57,59-61}$, thus while the guise of the sucrose crystal remained unaltered the habit tended towards prismatic.

The dextrans are usually removed from crude sucrose juices by the addition of binding agents $^{62,63}$, but the use of dextranase preparations is now being investigated $^{45,61,64,65}$.

Two major problems are encountered when enzymic degradation is employed. The first is the inactivation of the enzyme at the process temperatures $(60 - 70^\circ C)^{50,54}$. This is being overcome, at present, by using an enzyme system adsorbed onto an inert matrix $^{58}$. The second difficulty is the inability of most dextranases to degrade dextrans with relatively high degrees of branching $^{35,66}$.

Dextran is used in medical practice as a blood plasma expander $^{46-49}$, and two dextran preparations are in general use $^{67,68}$. 
Dextran 40 (\(M_w = 40,000\)) (a 10% w/v solution in dextrose or saline) inhibits red blood cell aggregation and lowers blood viscosity. It is used to overcome 'sludging of the blood' in which cell aggregates are formed which can plug arterioles and capillaries resulting in tissue anoxia and possible necrosis.

Dextran 110 (\(M_w = 110,000\)) (a 6% w/v solution in dextrose or saline) is administered as a temporary blood plasma substitute because its osmotic pressure is approximately equal to that of plasma proteins. Once infused, it is retained in the body for two to three days, a period long enough for the physiological replacement of plasma proteins. It helps to maintain venous return and it is also used in a prophylactic role in major surgery.

**JA2. Types and Percentages of Various Glucosidic Linkages in Dextrans**

Jeanes et al.\(^1\) have developed several techniques based upon infrared spectroscopy, the measurement of optical rotations, and periodate oxidation studies, involving the determination of the amount of periodate consumed and the amount of formic acid produced, for the elucidation of dextran structures, and these methods have been used for the analysis of a large number of dextrans\(^1,3,18,69-72\).

More exact information can be obtained by subjecting periodate oxidised dextrans to reduction and partial hydrolysis and analysing the products (Smith degradation). The results of several such studies are listed in table I 1.

Pasika and Cragg\(^73\) investigating the proton n.m.r. spectrum of \(O\)-deuterated \(L.\) mesenteroides NRRL B-512 and NRRL B-742 dextrans, and by comparison with the spectrum of \(O\)-deuterated isomaltotriose (I)
were able to assign peaks in the n.m.r. spectra at 4.95 τ to protons at C-1 associated with the α-(1→6) linkages and at 4.60 τ to protons at C-1 associated with the non-α-(1→6) linkages. Thus the ratio of the areas under the peaks at 4.95 τ and 4.60 τ gave the ratio of primary to secondary linkages.

Table 1 1 Types and Percentages of Differently Linked D-Glucopyranosyl Units in Some Dextrans as Determined by the Smith Degradation

<table>
<thead>
<tr>
<th>Dextran</th>
<th>% of D-Glucopyranosyl units linked (1→6) or (1→β)</th>
<th>(1→4)</th>
<th>(1→3)</th>
<th>(1→2)</th>
<th>References</th>
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<tr>
<td>L. mesenteroides</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>NRRL B-512</td>
<td>95</td>
<td>5</td>
<td></td>
<td></td>
<td>96</td>
</tr>
<tr>
<td>NRRL B-523S</td>
<td>93</td>
<td>3</td>
<td>4</td>
<td></td>
<td>96</td>
</tr>
<tr>
<td>NRRL B-742S</td>
<td>64</td>
<td>8</td>
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<td></td>
<td>96</td>
</tr>
<tr>
<td>NRRL B-742L</td>
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<td>4</td>
<td>trace</td>
<td></td>
<td>96</td>
</tr>
<tr>
<td>NRRL B-1064</td>
<td>95</td>
<td>2</td>
<td>3</td>
<td></td>
<td>96</td>
</tr>
<tr>
<td>NRRL B-1299S</td>
<td>56</td>
<td>7</td>
<td>36</td>
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<td>78</td>
</tr>
<tr>
<td>NRRL B-1299L</td>
<td>49</td>
<td>19</td>
<td>32</td>
<td></td>
<td>78</td>
</tr>
<tr>
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<td>53</td>
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<td></td>
<td></td>
<td>96</td>
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<td>NRRL B-1374</td>
<td>84.5</td>
<td>1</td>
<td>14.5</td>
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<tr>
<td>NRRL B-1415</td>
<td>87</td>
<td>12.5</td>
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</tr>
<tr>
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<td>7</td>
<td>10</td>
<td></td>
<td>76</td>
</tr>
<tr>
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<td>94</td>
<td>6</td>
<td></td>
<td></td>
<td>98</td>
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<td>Strain SF4</td>
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<td>44</td>
<td>71</td>
<td>29</td>
<td></td>
<td></td>
<td>70</td>
</tr>
<tr>
<td>S. mutans</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>strain G1:3 176</td>
<td>16</td>
<td>84</td>
<td></td>
<td></td>
<td>99</td>
</tr>
<tr>
<td>S. sanguis</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>strain 804</td>
<td>52</td>
<td>48</td>
<td></td>
<td></td>
<td>99</td>
</tr>
<tr>
<td>Tibi complex</td>
<td>90</td>
<td>1.5</td>
<td>8.5</td>
<td>6</td>
<td>6</td>
</tr>
</tbody>
</table>
Legend to structures

\[ \text{Legend to structures} \]

- or \( \text{R} \) continuation of a polysaccharide chain

- \( \text{O} \) \( \alpha\)-\( D\)-glucopyranose unit

- \( \text{OR} \) \( D\)-glucose reducing unit

\( \rightarrow \) \( \alpha\)-(1\( \rightarrow \)6) glucosidic linkage

\( \uparrow \) non-\( \alpha\)-(1\( \rightarrow \)6) glucosidic linkage

\( \uparrow \) glucosidic linkage resistant to enzymic hydrolysis

Table I 2 shows the results obtained when several dextran were investigated by this method.

Partial acid hydrolysis of dextran invariably yields the disaccharide isomaltose \( 1,18,74-78 \) and traces of kojibiose \( 78 \), nigerose \( 74 \), and maltose \( 76 \) have also been reported.

The failure to isolate the secondary-linked disaccharides from most dextran has been attributed to the relative instability of secondary linkages, as compared with primary linkages, to acid hydrolysis.
Fujimoto et al. found that the relative stabilities of the α-glucosidic linkages were reversed under acetalolysis conditions (possibly due to the steric hindrance of the solvated acetylium ion). Under such conditions, the disaccharides containing the secondary linkages have been obtained from many dextrans (table I 3).

Methylation studies have been utilised to determine the nature and proportions of the differently linked D-glucopyranosyl units in several native and clinical dextrans (table I 4).

Table I 2 Percentages of Secondary Glucosidic Linkages in Some Dextrans as Determined by Proton n.m.r. spectroscopy

<table>
<thead>
<tr>
<th>Dextran</th>
<th>% of Secondary-Linked D-Glucopyranosyl Units</th>
<th>References</th>
</tr>
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<tbody>
<tr>
<td>L. mesenteroides</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NRRL B-742</td>
<td>30</td>
<td>73</td>
</tr>
<tr>
<td>NRRL B-1355</td>
<td>33</td>
<td>100</td>
</tr>
<tr>
<td>NCIB 2706</td>
<td>20</td>
<td>101</td>
</tr>
<tr>
<td>S. mutans</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ingbritt</td>
<td>28</td>
<td>18</td>
</tr>
<tr>
<td>OZ 51</td>
<td>17-21</td>
<td>18</td>
</tr>
<tr>
<td>Dextran</td>
<td>Disaccharides</td>
<td>References</td>
</tr>
<tr>
<td>------------------</td>
<td>---------------</td>
<td>------------</td>
</tr>
<tr>
<td></td>
<td>Kojibiose</td>
<td>Nigerose</td>
</tr>
<tr>
<td></td>
<td>$\alpha-(1\rightarrow 2)$</td>
<td>$\alpha-(1\rightarrow 3)$</td>
</tr>
<tr>
<td>L. mesenteroides</td>
<td>L. mesenteroides</td>
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</tr>
<tr>
<td>NRRL B-512</td>
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<td>+</td>
</tr>
<tr>
<td>NRRL B-523</td>
<td>+</td>
<td>+</td>
</tr>
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<td>NRRL B-742L</td>
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<td>+</td>
</tr>
<tr>
<td>NRRL B-1149</td>
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<td>+</td>
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<td>NRRL B-1415</td>
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<td>NRRL B-1416</td>
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<td>NRRL B-1424</td>
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<tr>
<td>NRRL 44V-2</td>
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<tr>
<td>S. bovis strain 1</td>
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<td>S. mutans</td>
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<td>Ingbritt</td>
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<td>S. sanguis</td>
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<tr>
<td>ATCC 10558</td>
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<tr>
<td>S. viridians var.</td>
<td>S. viridians var.</td>
<td></td>
</tr>
<tr>
<td>NRRL B-1351</td>
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<tr>
<td>Streptobacterium</td>
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<td></td>
</tr>
<tr>
<td>dextranicum</td>
<td>+</td>
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</tbody>
</table>
The dextrans are methylated by one, or a combination, of the several available methylation procedures. Methylation studies have been conducted using the Haworth procedure\textsuperscript{6,19,40,74,81} the sodium-methyl iodide-liquid ammonia method\textsuperscript{76,78,81-83} and the Hakomori methylation procedure which uses dimethylsulphinyl carbamion and methyl iodide in dimethylsulphoxide solvent\textsuperscript{19,84-92}. The use of combined gas liquid chromatography-mass spectrometry (g.l.c.-m.s.) has greatly facilitated the separation and identification of the products of methylation analyses.

Examination of these results reveals several points of interest. All the dextrans possess some D-glucopyranosyl units linked through position 3, and the disaccharide nigerose is generally to be found in the acetylation product of dextrans. This fact has lead to the suggestion that the dextran-synthesising enzyme, dextransucrase, has the ability to transfer D-glucopyranosyl units to both the C-3 and the C-6 position\textsuperscript{93}. The α-(1→3) linkages in several dextrans occur at non-branch points. These elements of dextran structure can exist as occasional isolated α(1→3) linked units within the α-(1→6) dextran backbone, or as a section of contiguous α-(1→3) linked units within a predominately α-(1→6) chain or they may arise from a contaminating α-(1→3) linked glucan produced by the synthesising organism.

At least some of the α-(1→4) linked D-glucopyranosyl units that are occasionally detected in dextrans\textsuperscript{19,76,91,94} most probably arise from contamination of the dextran by intracellular polysaccharides of the starch-glycogen type, which are released into the culture medium when lysis of a substantial number of bacterial cells occurs \textsuperscript{17,19,95}. 
Table 14: Types and Percentages of Differently Linked D-Glucopyranosyl Units in Some Dextran as Determined by Methylation Analysis

<table>
<thead>
<tr>
<th>Dextran</th>
<th>% of D-glucopyranosyl units linked</th>
<th>Refs.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(1→2) (1→3) (1→4,6) (1→6)</td>
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<tr>
<td>L. mesenteroides</td>
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<tr>
<td>NRRL B-512</td>
<td>91</td>
<td>5</td>
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<td>NRRL B-512</td>
<td>91</td>
<td>4.5</td>
</tr>
<tr>
<td>NRRL B-742S</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>NRRL B-742L</td>
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<td>+</td>
</tr>
<tr>
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<tr>
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<td>S. sanguis</td>
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<tr>
<td>strain 804b</td>
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<td>52</td>
</tr>
<tr>
<td>Tibi complex</td>
<td>5</td>
<td>90</td>
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</table>

a This polysaccharide contains 8.0% of D-glucopyranosyl units linked (1→4).
b These polysaccharides were prepared from cell-free glucosyltransferase fractions.
A Structural Segments obtained from Dextran

Partial acid hydrolysis of dextran usually yields the disaccharide isomaltose and higher oligosaccharides of the isomaltose series (II).

Such oligosaccharides arise from contiguous α-(1→6)-linked D-glucopyranosyl units which comprise the dextran backbone. Disaccharides containing the secondary linkages have also been reported in the hydrolysates of a few dextran.

The characterisation of the oligosaccharides obtained from the acetolysates of dextran can provide valuable information about the nature of the branches in the polysaccharide.

The trisaccharides \(\alpha-D-(1\rightarrow6)\)-[\(\alpha-D-(1\rightarrow2)\]-D-glucose (III), \(\alpha-D-(1\rightarrow2)\]-\(\alpha-D-(1\rightarrow6)\)-D-glucose (IV) and \(\alpha-D-(1\rightarrow6)\)-\(\alpha-D-(1\rightarrow2)\]-D-glucose (V) have all been obtained from the acetolysate of Leuconostoc mesenteroides NRRL B-1397 dextran.
Trisaccharide (V) could arise from branches consisting of at least an isomaltosyl unit (VI) or from α-(1→2)-linked D-glucopyranosyl units occurring at non-branch points within the dextran chain (VII), and its presence in the acetolyate suggests the presence of one or both of these structural units in the B-1397 dextran.
Trisaccharide (V) along with the tetrasaccharide 2-α-D-glucopyranosyl-(1→6)-2-α-D-glucopyranosyl-(1→6)-2-α-D-glucopyranosyl-(1→2)-α-D-glucose (VIII) was identified in the acetolyzate of *L. mesenteroides* NRRL B-1299S dextran\(^{112}\). This suggests that some of the branches in the B-1299S dextran comprise at least an isomaltotrioseyl unit, and since all the α-(1→2) linkages form branch points a structure of the type (VII) can be excluded.

However a structure of type (IX) where the branch is essentially formed by a single α-(1→6)-linked D-glucopyranosyl unit, could still be present.
The absence of trisaccharide (V) in the product of the acetolysis and deacetylation of \textit{L. mesenteroides} NRRL B-1298$^{40}$ and the presence of trisaccharides (III) and (IV) are indicative of branches associated with the $(1\rightarrow2)$-branch points consisting of 2-O-$\alpha$-D-glucopyranosyl units.

The isolation of a trisaccharide containing only secondary linkages supports the existence of a structural segment in the dextran in which two secondary-linked-$\alpha$-D-glucopyranosyl units are adjacent.
Since the vast majority of the secondary linkages in the B-1298 dextran arises from branch points (table I 4) structural segments of the type (X) and (XI) are most likely.

Similar structural segments are suggested by the products of acetolysis and deacetylation of \textit{L. mesenteroides} NRRL B-1307 dextran\textsuperscript{104}.

The presence of a high proportion of secondary linkages in the \textit{L. mesenteroides} NRRL B-1355S dextran\textsuperscript{105}, the presence of trisaccharide (V) and the absence of trisaccharide (III) and the homologues of nigerose in the acetolysate of the B-1355S dextran provides evidence for an alternating type structure (XII).

(XII)

When subjected to the Smith degradation procedure, \textit{L. mesenteroides} NRRL B-1298 and NRRL B-1375 dextrans yielded both 1-\text{-}\textgreek{a}-isomaltosyl-glycerol (XIII) and 1-\text{-}\textgreek{a}-glucopyranosyl-glycerol (XIV)\textsuperscript{49,112}.
Since all the $\alpha-(1\rightarrow 3)$ linkages in the dextran form branch points, compound (XIII) could only arise from a segment of structure in which two adjacent $\alpha$-glucopyranosyl units both carried branches (XV).

On Smith degradation L. mesenteroides NRRL B-1355S, IFO 12370 and the Tibi complex dextrans gave principally compound (XIV)\(^6\), \(^98,105\). Thus the structural segment (XV) was not present in significant proportions in these dextrans.
Since nigerodextrinyl derivatives are absent from the products of the Smith degradation of most dextrans, segments consisting of contiguous α-(1→3)-linked β-D-glucopyranosyl units are deemed to be absent.

In comparison with the amount of work reported on the starch-glycogen-type polysaccharides, relatively few enzymic degradation studies have been carried out on dextrans.

Most of the work reported involves enzymes that degrade the α-(1→6) linkages of the polysaccharides (α-(1→6) glucan 6-glucanhydrolases [EC 3.2.1.11]). Such enzymes have been produced inductively, by growing bacteria or moulds on dextrans or dextran derivatives.

Exodextranases, i.e. enzymes that degrade the substrate in a stepwise fashion from the chain ends, have been obtained from species of soil bacteria, Bacillus, Bacteroides, Lactobacillus, Streptococcus, and from animal tissue, but little structural work involving such enzymes has been reported.

Endodextranases, i.e. enzymes that cleave dextrans to oligosaccharides in a random fashion have been obtained from Penicillium funiculosum, P. lilacinum, P. luteum, Aspergillus carneus, Lactobacillus bifidus and Cytophaga.

The enzymes produced by P. lilacinum, P. funiculosum, and by Lactobacillus bifidus have been studied by Weigel et al. These workers demonstrated that isomaltose (structure (II) n = 0) was the major product of hydrolysis of L. mesenteroides dextrans and that isomaltotriose (structure (II) n = 1) and a small amount of glucose were also produced along with limit dextrans. The limit dextrans
contained at least one secondary, non-\(\alpha-(1\rightarrow 6)\) linkage and the characterisation of the limit dextrans enabled the following pattern of resistant linkages to be established\(^{111,139}\).

The linkages in a dextran structure relatively resistant to attack by \textit{P. lilacinum} dextranase

\[
\begin{align*}
\text{\(\alpha-(1\rightarrow 6)\) linkage on the non-reducing side of a branch point will be resistant to the hydrolysis of \textit{P. lilacinum} dextranase but not resistant to that of \textit{P. funiculosum} dextranase.}
\end{align*}
\]
Thus, the smallest limit dextrins produced by the action of *P. lilacinum* and *P. funiculosum* dextranases acting on a branched dextran, in which all of the secondary linkages are $\alpha-(\rightarrow 3)$ linkages occurring at branch points, will be the pentasaccharide

$$\alpha-D-glucopyranosyl-(1\rightarrow 6)-[\alpha-D-glucopyranosyl-(1\rightarrow 3)]-\alpha-D-glucopyranosyl-(1\rightarrow 6)-\alpha-D-glucopyranosyl-(1\rightarrow 6)-D-glucose$$

(XVIII) and the tetrasaccharide

$$\alpha-D-glucopyranosyl-(1\rightarrow 3)-\alpha-D-glucopyranosyl-(1\rightarrow 6)-\alpha-D-glucopyranosyl-(1\rightarrow 6)-D-glucose$$

(XIX), respectively.

If however, the dextran contains a structural segment in which the secondary linkage does not form a branch point (VII) then on digesting the dextran with *P. lilacinum* dextranase, the smallest limit dextrin will be tetrasaccharide (XIX).
When acid-degraded dextrans were treated with the *Penicillium* enzymes, oligosaccharides were obtained which were not present in the hydrolysates of native dextrans. These may arise from either the acid-modified chain ends or from modified portions of the chains.

Sidebotham et al. characterised the pentasaccharides 

\[
\text{O-}^{\alpha-D-}\text{glucopyranosyl-(1} \rightarrow 2)\text{-O-}^{\alpha-D-}\text{glucopyranosyl-(1} \rightarrow 6)\text{-O-}^{\alpha-D-}\text{glucopyranosyl-(1} \rightarrow 6)\text{-D-glucose (XX)}
\]

and 

\[
\text{O-}^{\alpha-D-}\text{glucopyranosyl-(1} \rightarrow 6)\text{-O-}^{\alpha-D-}\text{glucopyranosyl-(1} \rightarrow 2)\text{-O-}^{\alpha-D-}\text{glucopyranosyl-(1} \rightarrow 6)\text{-D-glucose (XXI)}
\]

in the *P. lilacinum* dextranase digest of the acid-modified *L. mesenteroides* NRRL B-1299S dextran, and the authors suggest that these arise from unbranched acid-modified segments of the acid-degraded dextran, as in partial structure (VII).
As all the α-(1→2) linkages form branch points, in the native B-1299S dextran (table I 4) this structure (VII) must have been derived from a segment of the native polysaccharide as illustrated in partial structure (XXII).

Since statistical analysis of low molecular weight fragments obtained by partial acid hydrolysis of the native B-1299S dextran\(^{110}\) indicated that the branches consist principally of single D-glucopyranosyl groups, it follows that in partial structure (XXII) \(n = 0\).

The limiting factor in the use of the fungal enzymes is their inability to degrade highly branched dextrans\(^{35,66,89,140}\).

1A4 The Lengths of the Branches in Dextrans

The lengths of the branches in dextran molecules have been investigated by several groups of workers using different analytical techniques including physico-chemical studies\(^{141}\) statistical analysis of the products of acid hydrolysis\(^{110}\), modification and estimation of the non-reducing chain ends\(^{101,109,113,142}\) enzymic hydrolysis\(^{125}\),
132,134,143 and immunochemical studies 108,143,150.

By catalytic oxidation of the primary carbinol group on C-5 to a carboxyl group, the terminal glucosidic linkage in a chain can be stabilised with respect to acid hydrolysis, possibly due to the greater electronegativity of the carboxyl group 151.

Several possible non-reducing terminal sequences are shown as partial structures (XXIII)-(XXIX), along with the resulting oligosaccharide derivatives.

The structures of several native 109,113 and acid-degraded dextrans 113 have been examined by this procedure and from this work it has been concluded that at least 60% of the branches in L. mesenteroides NRRL B-1375 dextran and at least 50% of the branches in L. mesenteroides NRRL B-1415 dextran are of the type illustrated in partial structure (XXIII) 109.

The ratio of isomaltobiuronic acid and nigerobiuronic acid, obtained when L. mesenteroides NRRL B-512 dextran is catalytically oxidised and acid hydrolysed (4:1), considering the degree of oxidation (60%), indicates that at least 48% of the branches are as shown in partial structures (XXIV) and/or (XXV) and at least 12% of the branches are as shown in partial structure (XXIII) 109.

Another technique involving the modification of the terminal non-reducing chain ends has been employed by Rees et al 101 who replaced the primary carbinol groups in dextrans by O-(α-toluene-sulphonyl)hydroxymethyl groups and then identified the O-α-toluene-sulphonated oligosaccharides obtained on acid hydrolysis of the product.
This method was used to investigate the structure of \textbf{L. mesenteroides NCIB 2076 dextran} \textsuperscript{101} and it was concluded that the branches were principally of the type shown in partial structure (XXIV) but structural segments of the type (XXIII) and (XXV) were also present.

Lindberg and co-workers\textsuperscript{109,142} further modified this procedure by replacing the primary carbinol groups of the \textbf{L. mesenteroides NRRL B-512 dextran} by \textsuperscript{C-}(\textsuperscript{p}-toluenesulphonyl) methyl groups and then treating the modified dextran with alkali, and estimating the degree of branching of the product of methylation (or ethylation) analysis.

It was thus possible to successively degrade the modified dextran. The first degradation eliminated 40\% of the branches (which hence consist of a single \textsuperscript{\textbeta}-glucopyranosyl unit), the second an additional 45\% of the branches (which must have comprised two \textsuperscript{\textbeta}-glucopyranosyl units) and the remaining 15\% of the branches must have been three, or more, units long.

Further information on the length of the branch chains in dextrans can be obtained by enzymic degradation studies.

Rosenfeld et al\textsuperscript{131-135,128,129} isolated from animal tissue, an enzyme reported to cleave the \textalpha-\text((\textuparrow\textuparrow 6) linkages in dextrans in a stepwise fashion up to the branch points producing glucose and residual dextran only. There is, however, some evidence that linear \textalpha-\text((\textuparrow\textuparrow 3) linkages are also cleaved by the enzyme\textsuperscript{2,71,152}.

Reductions in molecular weight of 18-35\% have been reported for clinical dextrans treated with this enzyme\textsuperscript{134,151} and between 20-25\% for native dextrans\textsuperscript{129}.
The exodextranase [α-(1→6) glucosidase] isolated from a strain of *Streptococcus mitis* produced a reduction in molecular weight of 38% in an acid-degraded *L. mesenteroides* NRRL B-512 dextran and conversions to glucose of 22-27%, 34%, 24%, 17% 12% and 0% from the dextrans of *Streptococcus bovis* (various strains), a synthetic dextran, *Leuconostoc mesenteroides* NRRL B-512F dextran, *L. mesenteroides* NRRL B-1415 dextran, *L. mesenteroides* NRRL B-742 dextran and *L. mesenteroides* NRRL B-1355 dextran, respectively.

Such enzymic degradation studies allow the calculation of the minimum, average, non-reducing terminal sequence of D-glucopyranosyl units.

Further information concerning the non-reducing terminal D-glucopyranosyl units can be obtained from immunochemical studies.

Dextrans acting as soluble antigens are able to precipitate antidextran, antipneumococcal and antiteichoic acid antibodies. The extent of this reaction can be calculated from the amount of antibody protein (estimated as nitrogen) precipitated.

Inhibition studies in which oligosaccharides are allowed to compete for the available antibody sites have produced valuable information about the requirements of the combining sites of the antibody molecules. The antibody is generally directed against the D-glucopyranosyl units which form the non-reducing terminal sequences of dextrans.

Kabat et al. discovered that if purified dextrans were injected into animals or man antidextran antibodies were produced which were usually directed against α-(1→6) linked D-glucopyranosyl units but in a few individuals a mixture of antibodies, some of which were directed against non-α-(1→6)-D-
glucopyranosyl units were produced\(^{144-146}\).

The extent of the reaction of \(\alpha-(1\rightarrow6)\)-specific antibody towards fourteen different dextrans was shown to be proportional to the percentage of \(\alpha-(1\rightarrow6)\) linkages in the dextran, in most cases, and inhibition studies indicated that the most effective inhibitors, on a molar basis, were isomaltotetraose [structure (II) \(n = 2\)] and isomaltopentaose [structure (II) \(n = 3\)]. Thus, it was concluded that the antidextran is directed toward non-reducing, terminal sequences of 4 or 5 \(\alpha-(1\rightarrow6)\)-linked \(\beta\)-glucopyranose units\(^{147}\) [structure (XXX) \(n = 2\) or 3].

Similar studies using an \(\alpha-(1\rightarrow3)\) specific antibody indicated that the extent of the reaction was proportional to the percentage of \(\alpha-(1\rightarrow3)\) linkages in the dextrans\(^{144}\). The fact that \(L.\ mesenteroides\) NRRL B-1142 dextran showed little reaction was attributed to the occurrence of \(\alpha(1\rightarrow3)\) linkages within a chain (VII) rather than at terminal non-reducing positions in the polysaccharide\(^{1,144,148}\) (XXXI) and (XXXII).

When dextrans were tested with an \(\alpha-(1\rightarrow2)\)-specific antibody, those containing the greatest proportion of \(\alpha-(1\rightarrow2)\) linkages showed the greatest reaction\(^{144}\), indicating that many of the branches were terminated by kojibiosyl groups. Since the \(\alpha-(1\rightarrow2)\) linkages in \(L.\ mesenteroides\) NRRL B-1299 and NRRL B-1397 dextrans have been shown to occur only at branch points\(^{78,106}\), it follows that many of the branches in these dextrans consist of a single 2-O-\(\alpha-\beta\)-gluco-
pyranosyl unit (XXIII).
Certain dextrans have been shown to cross-react with pneumococcal types II, XX, XII, IX, and XIII antibodies and it has been shown that types II, XX and in some cases type XII antisera are directed against $\alpha-(\rightarrow 6)$ linkages.

Examination of a number of dextrans suggested that most reaction is obtained from the polysaccharides with the greatest proportion of $\alpha-(\rightarrow 6)$ linkages and oligosaccharide inhibition studies indicated that the antibodies are directed against terminal non-reducing sequences of at least 4 $\alpha-D$-glucopyranosyl units.

The capacity of dextrans to precipitate types IX and XIII pneumococcal antibodies was found to be proportional to the percentage of $\alpha-(\rightarrow 3)$ linkages in the polysaccharides and oligosaccharide inhibition studies have shown that these antisera are
directed against terminal non-reducing nigerosyl units. Such structures have been identified in the \textit{L. mesenteroides} NRRL B-1355S, NRRL B-1493S, NRRL B-1501S, NRRL B-1299S and NRRL B-1299L dextrans.

The pneumococcal type XII antibodies were shown to be directed against $\alpha(\text{1} \rightarrow \text{2})$, $\alpha-(\text{1} \rightarrow \text{3})$ and $\alpha-(\text{1} \rightarrow \text{4})$-linked non-reducing, terminal, glucopyranosyl units.\footnote{148,150}

The lectin, concanavalin A, obtained from \textit{Canavaria endoformis} and its reactions with native dextrans have been studied by Smith and co-workers\footnote{157} and by Goldstein \textit{et al.}\footnote{159,160} and it has been demonstrated that the extent of interaction of concanavalin A with native dextrans is dependent principally upon the number of terminal, non-reducing $\alpha$-$D$-glucopyranosyl units i.e. the degree of branching of the polysaccharide. Rosenfeld and Preobrazhenskaya\footnote{2} have also proposed a further modifying factor arising from the exclusion of the concanavalin A molecule by a highly branched dextran structure.

Concanavalin A will be precipitated by a dextran with a degree of branching of as little as 5% but not by a linear structure\footnote{108,159}. Thus, it could provide a rapid means of estimating the percentage of branching in dextrans.

By absorbing the concanavalin A onto sepharose gel, it is possible to separate a mixture of dextrans chromatographically according to their degree of branching and a product of this type is now being marketed\footnote{161}.

\textbf{1A5 The Biosynthesis of Dextran}

The dextran synthesising enzyme dextranucrase (E.C. 2.4.1.5) is a transglycosylase able to transfer $D$-glucopyranosyl units from the
Sucrose substrate to a growing $\alpha-(1\rightarrow6)$-linked $D$-glucopyranosyl chain. This characterisation of the enzyme is however far from complete, for dextranucrase is able to transfer $D$-glucopyranosyl units singly (or possibly in pre-formed blocks) to a variety of acceptor molecules from lactulosucrose and $\alpha$-$D$-glucopyranosyl fluoride in addition to sucrose.

\[(XXXIII)\]

The production of dextranucrase by strains of *Lactobacilli*, *Leuconostoc*, and *Streptococci* is reported, although in some cases the products of the biosyntheses have not been rigorously characterised. *Lactobacillus casei* (32-1) and *Lactobacillus RWM-13* are both reported to produce dextranucrases.

*Leuconostoc* species are widely reported to produce dextranucrase when cultured in a medium of which sucrose forms all or part of the carbohydrate source. Studies on the *Leuconostoc* dextranucrases have been hampered by the difficulty of obtaining dextran-free samples of the enzyme and this lead Hehre to postulate that the polysaccharide formed an integral part of the enzyme structure.
The majority of the strains produce culture soluble dextrans but a few also produce a culture-insoluble dextran. Smith studying such a strain, *L. mesenteroides* NRRL B-1299 using labelling techniques, showed that the majority of the culture-insoluble material consisted of an enzyme-dextran complex and he suggested that on completion of the synthetic cycle the dextran is released into the culture medium as a culture soluble polysaccharide.

Later studies however, on the purified polysaccharide, have indicated the presence of inherently insoluble polysaccharide in the *L. mesenteroides* NRRL B-1299 dextran.

The *Leuconostoc* dextranases generally exhibit optimum activity between pH 5-5.5 and at 29-34°C.

The *Streptococci* predominately produce a dextranase that is released into the culture medium although some examples of the structure-bound dextranases produced by species of *Streptococcus* have been reported.

Many of the strains of *S. mutans* studied produce, in addition to dextrans, extracellular polyfructans (possibly levans). In addition they tend to produce a significant number of non-branching α-(1→3)-linked D-glucopyranosyl units. It remains unclear if these linkages occur within a predominately dextran-type structure or if they form a separate polysaccharide of the type referred to as 'mutan' by Guggenheim.

The *Streptococcus* dextranases are constitutive enzymes, exhibiting optimum activity between pH 5-8.5 and at 37-45°C.
An examination of the results of chemical analyses of the various dextrans suggests that most dextrans can be considered as containing three distinct structural segments. The α-(1→6)-linked skeletal chains, branch points at which a β-glucopyranosyl unit is substituted at C-1, C-6 and at one secondary position, and portions of the dextran structure which comprise linear non-α-(1→6)-linked β-glucopyranosyl units, i.e. in which one (or more) β-glucopyranosyl unit(s) is substituted at C-1 and at one secondary position only.

To be totally satisfactory a mechanism of dextran biosynthesis must account for these three distinct types of structural segment.

In the enzymic synthesis of a dextran, the substrate, usually sucrose, becomes bound at the donor site of the dextransucrase molecule, the β-glucopyranosyl unit is split from the donor molecule, which is then released, and is transferred to the receptor molecule which is bound at the receptor site of the enzyme molecule.

Two different reactions are now possible. The β-glucopyranosyl-receptor complex can be split from the enzyme molecule (the multi-chain system) or it may remain bound to the enzyme and further β-glucopyranosyl units, being made available by the binding of further substrate molecules at the donor site of the enzyme, may be transferred to it (the single chain synthesis).

The failure to isolate oligosaccharides or low molecular weight dextrans in the early stages of synthesis are indicative of a single-chain type synthesis.

There are two distinct possible directions for the propagation of the skeletal chains of a dextran molecule. Glucopyranosyl groups can be transferred to either the reducing or the non-reducing end of
a growing dextran chain. Neely \textsuperscript{123} assuming that propagation occurred from the non-reducing end, as is usual for glycosyltransferase enzymes \textsuperscript{197}, proposed the following reaction sequence.

The sucrose and the acceptor molecule are bound simultaneously to the enzyme donor and receptor sites respectively. The glucopyranosyl group is transferred from the substrate to the acceptor with the release of a fructose molecule and further glucopyranosyl units are then transferred from a succession of sucrose molecules to the primary carbinol of the terminal non-reducing $D$-glucopyranosyl unit of the growing chain. Chain synthesis is terminated with the dissociation of the acceptor-enzyme complex.

Ebert \textsuperscript{189} assuming that chain propagation occurs from the reducing end suggested the following reaction sequence. A sucrose molecule enters the dextranucrase donor site, is hydrolysed, and the $D$-glucopyranosyl unit so released is transferred to the receptor site of the enzyme. A second substrate molecule then enters the newly vacated donor site, is hydrolysed, and the chain synthesis proceeds via the transfer of the $D$-glucopyranosyl unit to the $C$-1 position of the $D$-glucopyranosyl unit in the receptor site and the chain is propagated by a repetition of this sequence of reactions. Termination occurs with the dissociation of the dextran-enzyme complex.

Experimental evidence available, up to present, is insufficient to determine which of the two mechanisms in fact operates in dextran synthesis.

Early studies on the dextranucrase system suggested that the skeletal $\alpha-(1\rightarrow6)$ linkages and the non-$\alpha-(1\rightarrow6)$ linkages at the...
branch points, may have been produced by different enzyme systems\textsuperscript{123}, the 'branching enzyme' having a different ion requirement and heat stability\textsuperscript{195,198,199}. Further studies however failed to substantiate these findings\textsuperscript{34,81,124,185,189}.

Bovey\textsuperscript{195,198,199} suggested that branches consisting of a single $\beta$-glucopyranosyl unit might arise from the $\beta$-glucopyranosyl units of sucrose being transferred to secondary positions in the dextran chain and that long branches might arise from the scission and transfer of linear $\alpha(\rightarrow6)$-linked portions of the skeletal chains to secondary positions, by a second enzyme system.

This latter hypothesis arose from experiments in which Bovey found that dextrans continued to increase in molecular weight even after all the substrate (sucrose) had been exhausted. This, it was later pointed out\textsuperscript{201}, may have been due to association of the dextran molecules.

Ebert\textsuperscript{202} suggested that the formation of branches occurs when segments of dextran are transferred to secondary positions at the reducing ends of dextran acceptor molecules. Such a mechanism would however tend to produce a molecule in which the lengths of the branches are randomly distributed, whereas, in fact, most dextrans appear to contain a few long and a large number of very short branches.

A further mechanism suggested by Hehre\textsuperscript{197} proposed the building up of branches by the transfer of glucosyl units to the non-reducing chain ends of dextran chains at the secondary positions and the simultaneous propagation of branches by a single chain mechanism.

These findings would again yield a molecule which does not correspond with the known dextran branch length distribution.
Sidebotham\textsuperscript{203} suggested that the following mechanism appears to be the most likely. The short branches in dextran molecules arise as a result of glucopyranosyl transfer to secondary positions in the dextran chain, due to the non-total specificity of the dextran sucrase enzyme, and the branch chain is then propagated via a multichain mechanism. The longer chains must arise from the transfer of whole segments of $\alpha-(1\rightarrow6)$-linked $\beta$-glucopyranosyl units to secondary positions in the dextran chain.

**IA6 Conclusions**

Dextrans are polyglucans produced through the action of the family of enzymes known as dextranases, when a variety of micro-organisms are grown on sucrose-based substrates. They are composed of sequences of $\alpha-(1\rightarrow6)$-linked $\beta$-glucopyranosyl units, some of which carry branches at the C-3 position and possibly also at positions C-2 and/or C-4. Isolated (or isolated portions of) $\alpha-(1\rightarrow3)$-linked $\beta$-glucopyranosyl units may also occur, especially in water-insoluble polysaccharides.

The types and proportions of secondary linkages in a dextran are characteristic of that polysaccharide elaborated by a particular micro-organism, and the dextrans examined to date contain 2-33% of branching units.

The majority of the branches in the dextrans so far investigated comprise a single $\beta$-glucopyranosyl unit, but there is some evidence to indicate the presence of very long branches in some dextrans.
The most common structural segment occurring in dextrans is that represented by structure (II) and the structural unit (VI) is common to all dextrans so far investigated. Water-insoluble dextrans tend to contain a relatively high proportion of $\alpha-(1\rightarrow3)$ linkages which occur at other than branch points.
Diseases of the teeth and the supporting structures are the most widespread of all the diseases of man. Chronic periodontitis (pyorrhea) an inflammatory disease involving the gradual destruction of the supporting tissues of the teeth, is the principal cause of tooth loss, closely followed by dental caries, the familiar tooth decay, which is the major cause of tooth loss in subjects under the age of twenty. Both of these diseases appear to arise as the result of the presence in the oral cavity of various bacteria which produce a 'gelatinous felt-like mass' known as dental plaque, which covers, or partially covers, the tooth surfaces and occupies the gingival margins.

It was Van Leeuwenhoek approximately three hundred years ago, who first reported the presence of bacteria in the oral cavity, whilst developing the microscope. W.D. Miller, some two hundred years later, commented that the carious process appeared to occur as the result of the activity of micro-organisms at the carious lesions i.e. the points of attack of the disease, a view later endorsed by Black and Goadby. Black first reported the presence in the oral cavity of 'gelatine forming micro-organisms' and Goadby named one such organism Bacillus necrodentalis. It was this group of workers who first incorporated the term 'gelatinous microbic plaque' into the dental literature.

Oeskov first positively identified the caries-inducing bacteria as streptococci, so confirming an original proposition by Goadby.
and he reported the production by these micro-organisms of quantities of gelatinous polysaccharide.

Definitive proof that certain strains of micro-organism were agents in the caries process, came with the introduction of gnotobiotic (germ-free) animals into dental caries research. Early studies in this field were conducted by Orland et al.\textsuperscript{216-219}. When gnotobiotic rodents, which had been taken by Caesarian section and reared under totally germ-free conditions, were fed on a standard sterile diet, dental caries failed to develop, even on a microscopic scale, over a period of several months, indicating that caries could not develop in the total absence of bacteria. The subsequent introduction of certain strains of bacteria into the oral cavity of these animals produced extensive caries \textsuperscript{220-223}.

In 1960, Keyes\textsuperscript{224} discovered that one line of hamsters descended from a caries-active female that had been treated with penicillin to depress the cariogenic flora, was caries resistant. These animals remained caries resistant unless they became infected by being caged with non-resistant hamsters or by the introduction of cariogenic micro-organisms into their oral cavities\textsuperscript{225-227}.

Guggenheim\textsuperscript{228-230} demonstrated that when conventional animals (rats) were continually given erythromycin in their drinking water, in order to suppress the oral micro-flora, a state of relative gnotobiosis could be achieved. The introduction of a strain of an erythromycin-resistant bacterium into the oral cavity was then employed to demonstrate the establishment of the micro-organism in the oral cavity and its cariogenic potential.
In order to demonstrate the colonisation of the oral cavity under competitive conditions, Fitzgerald and Keyes\textsuperscript{231}, introduced an antibiotic-resistant strain of the micro-organism under investigation, into the oral cavity of a normal rodent. After a suitable period of time had elapsed samples of plaque were taken from various locations in the oral cavity and grown on the antibiotic-containing medium. Thus, the establishment and the sites on location of the colonies of the introduced micro-organism could be determined.

Using gnotobiotic techniques several micro-organisms have been shown to be cariogenic in mono-infected experimental animals (usually rats). These include \textit{Lactobacillus acidophilus}\textsuperscript{221}, \textit{L. casei}\textsuperscript{232}, \textit{Acetomyces viscosus}\textsuperscript{233,234}, \textit{A. naeslundii}\textsuperscript{234}, \textit{Streptococcus faecalis}\textsuperscript{235}, \textit{S. mutans OMZ 61}\textsuperscript{236}, \textit{S. mutans C67-1}\textsuperscript{237}, \textit{S. mutans D 282}\textsuperscript{238}, \textit{S. strain CF-71}\textsuperscript{222}, \textit{S. strain LM7}\textsuperscript{222}, \textit{S. strain PK1}\textsuperscript{222}, \textit{S. strain G3-5}\textsuperscript{222}, \textit{S. strain SS2}\textsuperscript{239}, \textit{S. strain SEB1}\textsuperscript{239}, \textit{S. strain D65}\textsuperscript{240}, \textit{S. strain D182}\textsuperscript{240} and a \textit{Streptococcus} resembling \textit{S. salivarius}\textsuperscript{241}.

Although gnotobiotic studies have, without doubt, been of great use in the investigation of the caries problem, care should be exercised in the interpretation of the results obtained.

The use of laboratory rodents has the obvious advantages of ease of handling and cheapness. However, the dentition of rodents is not identical with that of human beings and the coprophagous habits of rodents render them far from ideal animals for feeding studies.

Furthermore, the establishment of a particular strain of micro-organism in the oral cavity of a gnotobiotic rodent does not necessarily imply that the same micro-organism would become established \textit{in vivo} under conditions of competition.
The micro-organisms used to infect the gnotobiotic rodents are grown up initially in liquid culture and they have, in vitro, a mean doubling time of 0.5 hours, i.e. $10^9$ daughter bacteria result from a single bacterium after only 15 hours growth. Thus, ample scope for mutation of the culture exists in the course of an experiment.

Notwithstanding these limitations, gnotobiotic studies have demonstrated that one major requirement, for the production of dental caries, is the presence, in the oral cavity, of certain bacteria.

A further factor contributing to the production of dental caries is the type of diet consumed by the individual. The effect of food generally on the populations of the various oral micro-organisms has been reviewed by Bowen. The relationship between food intake and caries incidence can be most successfully demonstrated by studying either humans who are being fed by stomach tube or by tube-feeding laboratory animals. In such cases, a general fall in the population of lactobacilli results but the population of oral streptococci remains constant.

De Stoppelaar et al. demonstrated that at times of carbohydrate restriction the proportion of Streptococcus mutans fell to a very low level whereas that of S. sanguis rose. However, there is evidence that the metabolic activity of the plaque is greatly altered under conditions of drastically reduced oral food intake.

The eating of fibrous foodstuffs, as in primitive diets, markedly reduces the incidence of dental caries, and primitive tribes are found to harbour a wide variety of oral flora.
All the studies however suggest that little acid is produced by the dental plaque of primitive tribesmen even when a highly cariogenic diet is consumed for a short period of time. However, with the advent of civilisation to primitive areas, rampant caries is often seen to develop in the indigenous population.

The one dietary component most commonly cited as the major cause or cariogenesis is sucrose, common sugar. The evidence, largely circumstantial, has been reviewed by Newbrun, Winter and Leach, and is summarised below.

Sucrose is the major dietary sugar in advanced civilisations, where the incidence of caries is much higher than in primitive societies, but it is either absent from, or is only a minor component in, primitive diets.

If a selection of common dietary sugars are separately introduced into wax-stimulated saliva, or a suspension of dental plaque, then, on incubation, the sucrose-containing digest is found to produce copious quantities of tenacious material (polysaccharides). The micro-organisms which have been shown to produce dental caries in mono-infected animals also produce large quantities of sticky polysaccharide material when grown in a sucrose containing medium more so than in a medium containing other mono- or disaccharides.

More dental plaque is produced both in vitro and in vivo, on the smooth surfaces of the teeth, when sucrose is given as a dietary component rather than one of the other common dietary sugars.
Human clinical studies have shown that caries incidence is proportional to sucrose intake and furthermore, individuals with hereditary fructose intolerance, who are unable to consume any sucrose in their diets, have a much lower caries incidence than normal individuals.

Guggenheim\textsuperscript{264} however stresses that the total replacement of sucrose in the diet by other monosaccharides or disaccharides would not totally eliminate caries.

The third major factor associated with dental caries is the susceptibility of the host. Caries susceptibility is the term used to describe the relative, inherent or acquired predisposition of a person, an individual tooth or an individual tooth surface to dental caries\textsuperscript{265}.

Several workers, and groups of workers, have investigated the complex variations in tooth surface caries susceptibilities, and the reader is referred to publications by Parfitt\textsuperscript{266}, Barr et al.\textsuperscript{267}, Toverud et al.\textsuperscript{268}, Backer Dirks\textsuperscript{269}, Marthaler\textsuperscript{270} and Berman and Slack\textsuperscript{271}.

The caries susceptibilities of individuals differ widely, due partially to such conditions as tooth spacing and the depth and arrangement of the pits and fissures in the tooth surfaces. Food particles can become impacted in the pits, fissures and grooves in the occlusial surfaces (sulci) and cannot easily be removed by brushing\textsuperscript{272}. If, however, these imperfections in the tooth structure are filled with adhesive plastic, caries incidence is markedly reduced\textsuperscript{273}.

The rate of saliva flow also contributes to the caries susceptibility of an individual. In human beings, the condition of
xerostomia ('dry-mouth') is accompanied by rampant caries. Various human studies have shown a correlation between low saliva flow and a high caries experience and the surgical removal of the salivary glands of laboratory animals induced rampant caries when the animals were fed on a potentially cariogenic diet.

The eating habits of the host are also found to contribute to the hosts susceptibility to carious attack. Laboratory animals tend to develop more severe caries when allowed to feed ad libitum than do similar animals who are given larger meals of the same diet less frequently. A similar relationship between frequency of feeding and caries experience has been found in human studies.

The major requirements for the production of caries have been summarised diagrammatically by Scherp.

Figure 11  Scherp's representation of the host-parasite-environment complex as it affects the teeth
This diagrammatic representation serves to illustrate that all three of the basic requirements, a susceptible host, a source of fermentable carbohydrate and certain types of microflora must be present before dental caries can develop.

**The Development of the Dental Plaque and the Colonisation of the Tooth Surface by Oral Bacteria**

It is now generally recognised that dental plaque plays a major role in the aetiology of dental caries.

In the pits, fissures and grooves of the tooth surface, food can become impacted and especially in the occlusial grooves. This impacted food debris cannot be easily removed by brushing. On the smooth surfaces of teeth, however, such a mechanism cannot operate. Rather, the tooth surface becomes coated in dental plaque and the micro-organisms, which constitute a large part of the plaque, are able to metabolise dietary carbohydrates and parts of the complex organic matrix of the plaque to produce organic acids (the acidogenic theory of caries). This acid attacks and demineralises the enamel surface and carious lesions are produced.

It is proposed, in this section, to trace the development of dental plaque from the initial acquired pellicle stage to the final mature plaque.

The build up of oral plaque during periods of no oral hygiene has been studied by a number of workers.

The appearance of an electron dense cuticle covering the totally-cleaned surface of a tooth or an etched enamel surface within two hours of prophylaxis, has been demonstrated by Lenz and Muhlemann.
Theilade, Meckel, Frank and Brendel, Leach and Saxton, Armstrong, Loe et al and Jensen et al, using microscopic and bacteriological techniques.

This acquired pellicle is found to consist of a micro-organism-free cuticle, up to 15 microns in thickness, derived from proteins arising from the epithelial attachment lamina or from salivary glycoproteins, or both. Continuous with this is a subsurface cuticle which occupies the pits and fissures in the teeth and the interstitial spaces between the crystallites of the damaged enamel of the carious lesions. The acquired pellicle is continually reforming over all the available solid surfaces in the oral cavity and will even form over established plaque, eventually becoming covered by new microbial plaque, so producing lamination lines observed when the plaque is examined in section.

Within three days of prophylaxis, various micro-organisms begin to colonise the acquired pellicle surface. These comprise largely of gram-positive cocci. Associated with these micro-organisms, in the presence of the components of a normal diet, is a large quantity of extracellular polysaccharide material. This will be more fully discussed in Section 1B4.

After three days the number of gram negative rods and cocci increases and fusobacteria and filaments begin to appear.

After a period of approximately a week the gram-positive cocci and rods constitute about 60% of the total flora, the proportion of gram-negative cocci and rods and filaments and fusobacteria increase significantly and a few spirilla are to be detected. There is a tendency as plaque develops, for the proportion of anaerobic
micro-organisms to increase and the proportion of aerobic micro-
organisms to decrease.

After some three or four weeks the organisms in the lower layers
of the plaque die giving rise to ghostlike structures when viewed
through the electron microscope and the plaque matrix components
begin to crystallise giving rise to dental calculus.\(^{315}\)

The pattern of the production of dental plaque can be seen if
mature dental plaque or calculus is studied in section. Several
methods are available for the production of micro-thin sections of
deral plaque. The most usual method involves the fitting of
elemental glass slides,\(^{317}\) celloidin,\(^{318}\) agar wire\(^{320}\)
or plastic foils\(^{289,291,292,321}\) into prosthetic devices. The
prosthetic device is removed from the oral cavity after several days,
the plaque sample is sectioned, stained, and examined by visible or
electron microscopy. Some workers have used extracted human teeth
for such studies but the plaque in such cases is of uncertain origin
\(^{297,298,322-324}\).

Schroeder and De Boever\(^{325}\) have defined the structural components
characteristic of mature plaque.

The plaque-tooth interface consists of an essentially micro-
organism-free cuticle very similar to, but not identical to, the
acquired pelicle. Attached to this is the subsurface cuticle, a
fibrillar meshwork of salivary protein origin which spreads into the
intercrystalline spaces of eroded enamel.\(^{296,298,316}\) A third
component of the plaque-tooth interface is a series of fibrils of
thiosemicarbazide-osmium positive material continuous with the
plaque matrix. This material, bacterial extracellular water-insoluble
polysaccharide, is produced by the plaque micro-organisms. In some samples of mature dental plaque the proteinaceous cuticle is often absent and the bacterial polysaccharide itself is in direct contact with the tooth surface. Such a situation is almost invariably associated with severe erosion of the dental enamel.

The layer immediately above the cell-free cuticle has been termed the condensed microbial layer by Schroeder and Hirzel and it comprises the first colonies of micro-organisms to invade the tooth surface. These micro-organisms, predominantly streptococci, are characterised by a high number of cell dividing planes which lie parallel to the tooth surface. The condensed microbial layer consists of approximately 70% streptococci and 30% extracellular polysaccharide which occupies the narrow intercellular spaces.

After the first invasion of the tooth surface by colonies of streptococci further accumulations of microbial cells and extracellular material occur and it is this material that constitute the bulk of mature dental plaque. With these further accumulations, diversification of the microflora of the plaque occurs. Filamentous micro-organisms form a significant proportion of the plaque and these tend to lie with their long axes perpendicular to the tooth surface. The cocci and rods still constitute the major proportion of the plaque micro-organisms and small compact colonies of cocci can be observed when the plaque is studied in section. Around the microbial colonies and filling the intercellular spaces extracellular bacterial polysaccharides are to be detected. The polysaccharide is most dense around the coccoid micro-organisms and there is evidence
to suggest that the volume of bacterial polysaccharide is directly proportional to the intake of sucrose by the host\textsuperscript{261,331,332}.

Some of the intercellular spaces appear structureless and it is possible that these are empty spaces that were occupied by water-soluble polysaccharides which were leached out in the processing of the samples\textsuperscript{4,333,334}.

The appearance of the plaque surface varies morphologically depending on the proportion of the different micro-organisms in the plaque. The plaque surface of a filament-rich plaque is composed largely of filamentous micro-organisms interspersed by a few coccoid colonies. This gives the free surface a ridged appearance. The surface of a filament-poor plaque consists of coccoid organisms which form an even free surface. Associated with the free surface of both types of plaque is a large number of intercellular spaces and a large amount of slightly electron-dense extracellular polysaccharide\textsuperscript{335,336}.

IB3 Caries Prevention and the Control of Dental Plaque

Since the presence of the plaque deposits on the tooth is a factor \textit{sine qua non} for the production of smooth surface caries, it seems reasonable to assume that careful control of dental plaque will result in a decrease in caries incidence\textsuperscript{337}.

The most commonly applied method of plaque control is by mechanical tooth cleansing i.e. brushing. There are conflicting reports on the relationship between regularity of tooth brushing and the state of oral hygiene\textsuperscript{338–343} and tooth cleansing regimes based upon tooth brushing twice or three times a day are scientifically unfounded.
Loe et al. have demonstrated that thorough brushing once every second day is sufficient to maintain clinically healthy gingivae and teeth provided that the recommended brushing technique is employed. With a small application of time, technique and effort it is possible to maintain adequate plaque control by simple mechanical means.

Initial biochemical methods of plaque control involved the topical application of crude pancreatic enzyme preparations and mucinases to the teeth. When certain strains of oral streptococci were shown to be able to elaborate dextrans which formed a major part of the plaque (see Section 1B4) fungal dextranases were investigated for their ability to act as plaque-controlling agents. Initial investigations on animals, using dextranase incorporated into their drinking water and food, and using artificial plaque were successful in reducing the amount of plaque and reducing sucrose-induced agglutination of streptococcal cells, but later clinical studies, using highly purified enzymes, were unsuccessful.

There are several possible explanations for the failure of dextranases to break down the plaque matrix in vivo.

(i) The enzyme preparations used for the early work were relatively impure and part of the plaque controlling ability may have been due to contaminating proteases and carbohydrases.

(ii) The plaque produced in animals mono-infected by a single strain of plaque micro-organism (this was the model system used in some of the early investigations) differs substantially from that produced in vivo by the indigenous microflora.
(iii) It has been demonstrated that fungal dextranases are unable to degrade highly-branched dextran structures once the polysaccharide is constituted.

(iv) The dwell time of the enzyme within the oral cavity is too short for a significant amount of glucan degradation to occur.

(v) The plaque glucan is not necessarily a simple dextran but may contain polysaccharides of the 'mutan' type.

(vi) The conditions in the oral cavity are not the optimum for dextranase activity.

As a result of these difficulties much of the interest in carbohydrases as plaque-controlling agents has declined.

Clinical investigations have suggested that teeth exposed to natural fluoride are cleaner than teeth of subjects living in non-fluoride areas and the incidence of dental caries is greatly diminished.\(^\text{361}\).

Laboratory experiments have indicated that fluorine concentrations of more than 250 p.p.m. prevent bacterial growth and topical applications of fluoride in animal trials have been shown to reduce plaque count.\(^\text{361,362}\).

Rather than reducing the bacterial growth, it is more probable that fluoride reacts with the enamel, lowering the surface energy of the teeth, and reducing the ability of the enamel to adsorb proteins and polysaccharides.\(^\text{363,364}\).

Since the dental plaque is essentially a bacteriological system it follows that an antibiotic agent would drastically reduce the plaque micro-flora and hence the dental plaque. Penicillin, tetracyclin, spiramycin and varicomycin, in various formulations,
have been shown to be effective as plaque-controlling agents in laboratory animals$^{362, 365-367}$ and in humans, when applied for the treatment of ulcerative gingivitis and chronic periodontal disease$^{368, 369}$.

However, there is no doubt as to the potential danger of maintaining a continuous antibiotic regimen. Antibiotic sensitisation of a patent can result and the risk of producing resistant strains of bacteria would be very high.

Various non-antibiotic, antibacterial agents have been assessed for the use in dentifrices and mouthwashes with limited success$^{206, 328, 313, 370}$. But since the nature of the ecological shift, that antimicrobial agents (including antibiotics) would necessarily produce is not fully understood, it is unlikely that such material will be introduced for clinical use$^{371}$.

The possibility of vaccinating caries susceptible individuals against $S. \text{ mutans}$, which are considered the major bacterial cariogenic agent, has recently received much interest$^{372-377}$.

Animals injected with whole streptococci have been shown to produce antidextranucrase antibodies which inhibit dextran synthesis$^{372, 374, 378}$. This procedure is not, however, acceptable in human clinical studies because of biochemical side effects. If human subjects are injected with purified $S. \text{ mutans}$ dextranucrase, however, they are found to produce antidextranucrase antibodies without the biochemical complications$^{379}$.
Dental plaque is widely considered to be the key factor in dentogingival pathology. It is characterised by the high density of micro-organisms that it contains, by its potential for the production of organic acids from fermentable carbohydrate sources and by its ability to synthesise large amounts of extracellular polysaccharides.

The polysaccharide matrix appears to perform several functions. Van Houte et al have demonstrated that the tenacious polysaccharide produced by some strains of oral micro-organism, notably *Streptococcus mutans* and *S. sanguis* enables them to colonise the tooth surface soon after prophylaxis, and these workers showed that the ability of certain oral streptococci to colonise, not only the cleaned tooth surface but also the plaque surface and the surface of human and animal epithelial cells was related to their ability to produce this adhesive extracellular material.

In established plaque the insoluble extracellular polysaccharide forms a stable matrix in which the oral micro-organisms can exist without the danger of being washed from the tooth surface by the saliva flow of the host, and also it acts as an agent in the agglutination of streptococcal cells to form compact colonies.

The plaque matrix further acts as a semi-selective diffusion barrier allowing small molecules to diffuse to the tooth surface but not allowing the polysaccharides elaborated by the plaque microflora to diffuse out.

Finally, part of the water soluble fraction of the plaque polysaccharide is apparently able to act as a reserve carbohydrate at times of food depletion.
Investigations into the nature of the polysaccharide material that constitutes approximately 10% of the dry weight of the plaque have been performed by several groups of workers\textsuperscript{35,366}. In general, two methods of approach have been adopted. The first involves the collection of quantities of dental plaque from a large number of subjects. The plaque is then pooled and the polysaccharide component is extracted and analysed. The alternative approach involves the isolation of a single strain of oral micro-organism. This is either used directly to elaborate polysaccharide \textit{in vitro} or the polysaccharide-synthesising enzymes produced by the micro-organism are isolated, purified, and used to produce polysaccharide \textit{in vitro}.

Several workers conducting pooled plaque studies have demonstrated that a large proportion of the plaque carbohydrate is dialysable\textsuperscript{384-386}. Hotz \textit{et al}\textsuperscript{386} analysed the dialysable material and found it to comprise principally hexose and disaccharide along with some oligosaccharides and unidentifiable material and the authors suggest that this material arises from the action of plaque hydrolases on plaque glucans.

Wood \textit{et al}\textsuperscript{33,334,383} were able to extract the polysaccharide component of the plaque with water and alkali and the subsequent acid hydrolysis of the extracted polysaccharides showed glucose to be the major, and fructose the minor, component of the water soluble material. Wood\textsuperscript{383} also demonstrated that the quantity of water-soluble material fell markedly if whole plaque was incubated in the absence of external carbohydrate indicating that at least part of
the water soluble material was able to function as a carbohydrate reserve.

Leach et al.\textsuperscript{387-389} also demonstrated the presence, in dental plaque, of both glucose- and fructose-containing polymers and the lability of the latter. Since the fructose containing polymers, which are reported to constitute up to 20\% of the polysaccharide fraction of some plaques\textsuperscript{383}, are water soluble, even at very high molecular weights, it is improbable that they play any structural role in the plaque matrix but rather function solely as a reserve.

Hotz et al.\textsuperscript{386} analysed the pooled plaque obtained from 3,500 Zurich school children and were able to demonstrate the presence of not only glucose-containing plaque polysaccharides, which formed the majority of the plaque polysaccharide, but also the presence of heteropolysaccharides containing pentoses and other hexoses. A relatively high percentage of the glucan (equivalent to 1.35\% of the total plaque dry weight) was shown to consist predominantly of \(\alpha-(\longrightarrow 3)\)-linked \(\beta\)-glucopyranosyl units.\textsuperscript{386} This material has been termed 'mutan' by Guggenheim\textsuperscript{99} and it is proposed that this material performs an essentially structural role in the plaque, forming a rigid polysaccharide matrix. The isolation of a polysaccharide produced by single strain of streptococcus was first described by Oeskov and Paulsen in 1931. The micro-organism, isolated from the pharynx was later identified as \textit{Streptococcus salivarius} by Niven et al.\textsuperscript{390,11} and the polysaccharide elaborated by this micro-organism was identified as levan.
Garszynski and Edwards\textsuperscript{391} have described the production and subsequent partial analysis of a broth levan by \textit{S. salivarius} SS2.

A polysaccharide-synthesising enzyme described by the author as levansucrase \([\beta-2, 6\text{-fructan:}\text{D-glucose:}6\text{-fructosyltransferase (E.C. 2.4.1.10)}]\) has been isolated from \textit{S. mutans} JC2 by Carlsson\textsuperscript{193} and the production of a fructan \((M_w > 25 \times 10^6)\) by this enzyme has been demonstrated.

Lancefield group H \textit{Streptococci}\textsuperscript{20} and \textit{Streptococcus} \textit{s.b.e.}\textsuperscript{392} both of oral origin, were shown to produce an extracellular polysaccharide similar to dextran.

Guggenheim et al prepared several transferase enzymes from strains of \textit{S. sanguis}\textsuperscript{187} and \textit{S. mutans}\textsuperscript{34,99}. The polysaccharides elaborated by the transferases of \textit{S. sanguis} on incubation with sucrose, were shown to be essentially dextran-like in nature, whereas the polysaccharide elaborated by one of the transferases obtained from \textit{S. mutans} was shown, by methylation techniques, to contain 94\% of \(\alpha-(1\rightarrow 3)\)-linked \textit{D}-glucopyranosyl units and hence is a 'mutan'-type polysaccharide\textsuperscript{89}.

Sidebotham et al\textsuperscript{18} showed that the extracellular polysaccharides produced by whole cell cultures of \textit{S. mutans} and \textit{S. sanguis} grown \textit{in vivo} were essentially dextrans containing up to 30\% of non-\(\alpha-(1\rightarrow 6)\) linkages.

Edwards et al\textsuperscript{19,91,393} succeeded in analysing the polysaccharides produced \textit{in vivo} by several strains of \textit{S. mutans}. The glucans produced consisted of up to 30\% of \(\alpha-(1\rightarrow 3)\)-linked \textit{D}-glucopyranosyl units and up to 80\% of \(\alpha-(1\rightarrow 6)\)-linked \textit{D}-glucopyranosyl units. In the case of \textit{S. mutans} E49, the
polysaccharide was shown to contain 13% of $\alpha-(1\rightarrow 4)$-linked D-glucopyranosyl units and it is likely that these originated from contaminating intracellular material liberated into the culture medium when bacterial cells are allowed to lyse.

Baird and Ellwood, in investigating the polysaccharide elaborated by *S. mutans* Ingbritt, demonstrated that the glucan contained 51% of $\alpha-(1\rightarrow 3)$-linked D-glucopyranosyl units and 27% of $\alpha-(1\rightarrow 6)$-linked D-glucopyranosyl units. Two di-O-methyl-D-glucitol derivatives were detected in the acetylated, reduced, hydrolysed, methylation mixture (molar percentage 12%), but neither were characterised.

Evidence has already been presented for the breakdown of soluble dextran by the plaque micro-flora. Wood showed that under conditions of reduced carbohydrate intake the amount of soluble plaque polysaccharide falls by up to 50% and since the author had already demonstrated that the majority of this material is soluble dextran it follows that it forms part of the polysaccharide reserve along with the intracellular glycogen-type polysaccharide and the extracellular fructan.

This is further supported by the presence in pooled human plaque of large quantities of low molecular weight carbohydrates, the possible enzymic breakdown products of plaque glucanohydrolases acting on the plaque dextrans. However, it should be noted that the mixed plaque microflora and single strain *Streptococci* are reported to be unable to metabolise high molecular dextrans.
The dental plaque is a microscopic ecosystem involving many complex enzyme-induced interactions\(^{396}\). The ability of the plaque to synthesise large quantities of extracellular fructan and glucan has already been discussed. The caries process is, however, basically dependent upon the ability of the plaque micro-flora to produce organic acids, principally lactic acid, which attacks the enamel surface, producing carious lesions and eventually demineralising the tooth surface. The major acidic metabolic product of the dental plaque has been shown to be lactic acid\(^{301,302,397-402}\) but the production of formic acetic and butyric acids has also been demonstrated\(^{91,402,403}\). Acid production can occur through several routes. Wood\(^{383}\) has demonstrated the metabolism of soluble plaque glucans to yield acid metabolites. Possibly more significant is the breakdown of high molecular weight plaque fructans by the plaque micro-flora.

Fructan hydrolysis by plaque micro-organisms has been investigated by several workers\(^{4,39,97,203,334,404-406}\) and Manly \textit{et al}\(^{394}\) have noted that samples of fasting plaque contained little or no fructan as a consequence of this catabolism.

The levanhydrolyase of an oral streptococcus has been studied in greater detail by Da Costa and Gibbons\(^{404}\) and was shown to be an inducible enzyme that was present in the culture supernatant and in washed cell suspensions. It was shown to hydrolyse both levan and inulin and had a maximal activity at pH 6.0.

There is, however, a third biochemical route for acid production in dental plaque. Robrish and Krichevsky\(^{380}\) demonstrated that the yield of acid per molecule of sucrose was the
same as might be expected from two hexose units. This suggests that little of the available sucrose is diverted to the production of extracellular polysaccharide and the majority is accounted for as acidic fermentation products. Tanzer et al., using radioactive labelling techniques, were able to demonstrate the presence of three distinct enzyme systems in dental plaque.

(i) An α-1,6 glucan : \(\beta\)-fructose 2-glucosyltransferase
   (E.C. 2.4.1.5) (dextran sucrase)

(ii) A β-2,6-fructan: \(\beta\)-glucose 6-fructosyltransferase
    (E.C. 2.4.1.10) (levan sucrase)

(iii) A third enzyme system which is able to split sucrose independent of the transferases and allows the fermentation of the glucosyl and fructosyl moieties (an invertase-type enzyme) and such an enzyme has been isolated from dental plaque and characterised.

It is perhaps surprising, in view of the importance of plaque glucan in the aetiology of dental caries, that, to date, no detailed investigation has been conducted into the structure of the glucose containing polymers elaborated by oral streptococci. The aim of the present work is to investigate in detail the structure of polysaccharides produced in vitro by two strains of Streptococcus mutans.
Chapter II  THE POLYSACCHARIDE ELABORATED BY STREPTOCOCCUS

MUTANS GS-5

IIIA Preparation and Extraction

The organism Streptococcus mutans GS-5 was kindly donated by Dr. W.H. Bowen of the Royal College of Surgeons of England.

It was recovered from the lyophilised state, maintained, and the polysaccharide was produced as described (VI A 1). The resulting polysaccharide was fractionated by ethanolic precipitation. One polysaccharide fraction (GS-5 A polysaccharide) was precipitated with 40% ethanol (v:v) and the other polysaccharide fraction (GS-5 B polysaccharide) was precipitated with 70% ethanol (v:v). The crude polysaccharide fractions were purified by deproteination and repeated ethanol precipitation (VI A 1) and the yields of the freeze-dried fractions for two separate preparations are shown in table II 1.

Table II 1  The yields of GS-5A and GS-5B polysaccharides

<table>
<thead>
<tr>
<th>Batch</th>
<th>Yield in g of fraction A from 100 g of sucrose</th>
<th>Yield in g of fraction B from 100 g of sucrose</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.06</td>
<td>0.81</td>
</tr>
<tr>
<td>2</td>
<td>0.50</td>
<td>0.73</td>
</tr>
</tbody>
</table>

These yields are of the same order of magnitude as those obtained from various strains of S. mutans grown under similar conditions.
IIB  The GS-5 B polysaccharide

IIB 1  Determination of the monosaccharide components by acid hydrolysis

It has been widely reported that certain strains of _S. mutans_ produce polymeric species containing fructose in addition to glucose-containing polymers, when grown on a sucrose medium. Investigation of the monosaccharide component of the GS-5 B polysaccharide was thus necessitated. Two hydrolysis experiments were performed. One at the optimum conditions for the production of glucose from a glucose-containing polysaccharide, and one at the optimum conditions for the production of fructose from a polyfructan.

(a) **Hydrolysis at the optimum conditions for the production of glucose**

A small quantity of the polysaccharide was hydrolysed under conditions (1.0 M sulphuric acid, 100°C, 8 h) designed to completely cleave glucosidic linkages in the polysaccharide with the minimum degradation of the glucose released (VIA 2). The chromatographic properties of the hydrolysate and the reduced hydrolysate are shown in table VI 1. The hydrolysate, when subjected to chromatography in solvent (d) and electrophoresis in buffer (a), gave a single spot migrating as glucose, and the reduced hydrolysate, when subjected to electrophoresis in buffer (b) gave a single spot migrating as glucitol.
(b) Hydrolysis at optimum conditions for the production of fructose

A small quantity of the polysaccharide was hydrolysed under mild conditions (0.1 M sulphuric acid, 70°C, 1 h) designed to completely cleave fructosidic linkages with the minimum degradation of the fructose released (VIA 3). The chromatographic properties of the hydrolysate and the reduced hydrolysate are tabulated in table VI 2. The hydrolysate when subjected to chromatography in solvent (d) and electrophoresis in buffer (a) gave a major spot migrating as fructose and a minor spot migrating as glucose. The major spot stained with staining reagent (e), which is specific for ketohexoses. The reduced hydrolysate when subjected to electrophoresis in buffer (a) gave two spots migrating as glucitol and mannitol (the reduction products of fructose).

IIB 2 Composition

It has been demonstrated (IIB 1) that glucose and fructose were the only monosaccharide components to be detected in the hydrolysates of GS-5 B polysaccharide.

Quantitative estimation of these and other, non-carbohydrate, components was carried out as described (VIA 4).

The results are tabulated in table II 2.

Table II 2 Composition of GS-5 B polysaccharide

<table>
<thead>
<tr>
<th>Carbohydrate content</th>
<th>Glucose content</th>
<th>Fructose content</th>
<th>Protein content</th>
<th>Ash content</th>
<th>[α]D20 (c 0.995, M NaOH)</th>
</tr>
</thead>
<tbody>
<tr>
<td>%</td>
<td>%</td>
<td>%</td>
<td>%</td>
<td>%</td>
<td>+169°</td>
</tr>
<tr>
<td>97</td>
<td>93.4</td>
<td>2.5</td>
<td>1.5</td>
<td>0.85</td>
<td></td>
</tr>
</tbody>
</table>

From these results, it can be seen that GS-5 B polysaccharide is principally a glucose-containing polysaccharide.
IIB 3. Determination of the ratio of primary and secondary linkages by nuclear magnetic resonance spectroscopy (n.m.r.)

Pasika and Cragg\textsuperscript{73} examined the spectra of the dextrans produced by Leuconostoc mesenteroides NRRL B-512 and \textit{L. mesenteroides} NRRL B-742 after deuteration, and their work suggested that n.m.r. spectroscopy could provide a rapid method for determination of the ratio of glucose units linked \(\alpha-(1\rightarrow6)\) to those linked non-\(\alpha-(1\rightarrow6)\). The main difficulty encountered in such studies is, however, the relative insolubility of some dextrans in deuterium oxide\textsuperscript{18,78}.

Using deuterated isomaltotriose as a model compound, Pasika and Cragg\textsuperscript{73} were able to assign peaks at \(\tau = 6.30\) p.p.m. to protons on carbons C-5 and C-6, those at \(\tau = 6.10\) p.p.m. to protons on carbons C-2, C-3 and C-4, and those at \(\tau = 4.95\) p.p.m. to protons at C-1 involved in \(\alpha-(1\rightarrow6)\) linkages. In addition peaks at \(\tau = 5.33\) p.p.m. and 4.71 p.p.m. were assigned to protons attached to anomic carbon atoms not linked to other glucopyranosyl units.

The proton n.m.r. spectrum of deuterated \textit{L. mesenteroides} NRRL B-512 dextran contained only peaks at \(\tau = 6.30, 6.10\) and 4.95 p.p.m. The peaks due to protons attached to anomic carbon atoms not linked to other glucopyranosyl units were absent (these becoming statistically insignificant as the molecular weight becomes large).

Investigation of the dextran elaborated by \textit{L. mesenteroides} NRRL B-742, which contains 28\% of \(\alpha-(1\rightarrow3)\) linkages\textsuperscript{96}, indicated the presence of three peaks similar to those of the B-512 dextran, but in addition a peak at \(\tau = 4.60\) p.p.m. was present. This was assigned to
protons attached to carbons associated with the non-α-(1→6)
linkages (in this case α-(1→3) linkages) and arises from the
protons attached to either C-3 or C-1.

Since it was demonstrated that this peak arises at the expense
of that at ω = 4.95 p.p.m., it must be due to the protons attached to
C-1 involved in the non-α-(1→6) linkages.

Thus, integration of the areas under the peaks at ω = 4.95 p.p.m.
and ω = 4.60 p.p.m. will give the ratio of glucose units linked
α-(1→6) (primary linked units) to those linked non-α-(1→6)
(secondary linked units).

The peak assignments of the L. mesenteroides NRRL B-742 dextran
are shown in table II 3).

<table>
<thead>
<tr>
<th>Peak</th>
<th>δ p.p.m.</th>
<th>ω p.p.m.</th>
<th>Peak assignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>-3.70</td>
<td>6.30</td>
<td>C-5 and C-6 protons</td>
</tr>
<tr>
<td>2</td>
<td>-3.90</td>
<td>6.10</td>
<td>C-2, C-3 and C-4 protons</td>
</tr>
<tr>
<td>3</td>
<td>-4.80</td>
<td>5.20</td>
<td>DHO</td>
</tr>
<tr>
<td>4</td>
<td>-5.05</td>
<td>4.95</td>
<td>C-1 protons associated with α-(1→6)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>link.</td>
</tr>
<tr>
<td>5</td>
<td>-5.40</td>
<td>4.60</td>
<td>C-1 protons associated with non-α-(1→6)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>link.</td>
</tr>
</tbody>
</table>

Reference compound tetramethylsilane

Sidebotham et al have used this technique to investigate the
polysaccharides elaborated by L. mesenteroides NRRL B-129978 and
several strains of Streptococcus mutans 18.
GS-5 B polysaccharide was deuterated and the n.m.r. spectrum obtained (VIA 5). The spectrum is shown in figure VI 3 and the peak assignments in table II 4.

**Table II 4 Peak assignment of the n.m.r. spectrum of deuterated GS-5 B polysaccharide**

<table>
<thead>
<tr>
<th>Peak</th>
<th>δ (p.p.m.)</th>
<th>τ (p.p.m.)</th>
<th>τ corrected to t.m.s.</th>
<th>Assignment of peaks</th>
<th>Integration</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>-3.87</td>
<td>6.13</td>
<td>6.29</td>
<td>C-5 and C-6 protons</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>-4.06</td>
<td>5.94</td>
<td>6.10</td>
<td>C-2, C-3 and C-4 protons</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>-5.03</td>
<td>4.97</td>
<td>5.13</td>
<td>HDO</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>-5.22</td>
<td>4.78</td>
<td>4.94</td>
<td>C-1 protons associated with α-1→6 linkage</td>
<td>7.85</td>
</tr>
<tr>
<td>5</td>
<td>-5.56</td>
<td>4.44</td>
<td>4.60</td>
<td>C-1 protons associated with non α-1→6 linkage</td>
<td>1.55</td>
</tr>
</tbody>
</table>

The standard used was an external standard of hexamethyldisilane (h.m.d.s.), and as this has a resonance slightly shifted from that of tetramethyilsilane (t.m.s.) (0.16 p.p.m.) a correction had to be applied.

The integration of the area under the peaks τ = 4.94 and τ = 4.60 gives a ratio of primary to secondary linkages of 2:5:1.

**IIB 4 The infrared spectrum of GS-5 B polysaccharide**

Burket et al. observed that various dextrans, in their infrared spectra, showed differing amounts of absorption at 794 cm⁻¹. This was later attributed, by correlation with the results of periodate oxidation studies, to the varying proportions of secondary linkages in the dextran molecules.
Barker et al.\textsuperscript{14}, by investigation of a series of polysaccharides and oligosaccharides, were able to show that valuable information could be gained about glucan structures by studying the infrared spectra of the glucans in the region $1000 \text{ cm}^{-1} - 600 \text{ cm}^{-1}$.

The infrared spectrum of GS-5 B polysaccharide (figure VI 4) was obtained as described (VIA 6). The assignment of the peaks is shown in table II 5.

Table II 5  Peak assignment of the i.r. spectrum of GS-5 B polysaccharide

<table>
<thead>
<tr>
<th>Peak</th>
<th>Wave No. $\text{cm}^{-1}$</th>
<th>Assignment</th>
<th>Literature value $\text{cm}^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>909</td>
<td>$\alpha-(\uparrow \rightarrow 6)$ linked glucose units</td>
<td>916</td>
</tr>
<tr>
<td>2</td>
<td>838</td>
<td>$\alpha-D$-glucopyranose</td>
<td>838</td>
</tr>
<tr>
<td>3</td>
<td>780</td>
<td>$\alpha-(\uparrow \rightarrow 3)$ linked glucose units</td>
<td>788</td>
</tr>
<tr>
<td>4</td>
<td>756</td>
<td>$\alpha-(\uparrow \rightarrow 6)$ linked glucose units</td>
<td>768</td>
</tr>
<tr>
<td>5</td>
<td>711</td>
<td>nujol</td>
<td></td>
</tr>
</tbody>
</table>

This evidence suggests that GS-5 B polysaccharide contains $D$-glucopyranose units linked $\alpha-(\uparrow \rightarrow 6)$ and $\alpha-(\uparrow \rightarrow 3)$.

IIB 5  Types and percentages of the variously linked $D$-glucose residues in GS-5 B polysaccharide as determined by methylation analysis

The methylation analysis of a polysaccharide can be considered as a three step operation. The first step consists of the complete methylation of the polysaccharide, converting all the hydroxyl groups to methoxyl groups, the second step is the hydrolysis of all the
glycosidic linkages in the methylated polysaccharide to yield the partially-methylated monosaccharides and the subsequent modification of the partially-methylated monosaccharides. The third step is the separation and characterisation of the modified partially-methylated monosaccharides.

By this technique it is possible to determine both the types, and the percentages of the various types of linkage in a polysaccharide.

Several methods are available for the methylation of a polysaccharide but probably the most efficient and facile is that involving dimethylsulphinyl carbamion (figure II 1) in dimethylsulphoxide.

Figure II 1 Function of dimethylsulphinyl carbamion in the Hakomori methylation procedure

\[
\begin{align*}
\text{R-OH polysaccharide} + H_3C-S-CH_2^- Na^+ &\rightarrow R-O^- Na^+ + H_3C-S-CH_3^+ \\
\text{Sodium dimethyl-} &\text{ polysaccharide sulphinyl carbamion} \\
\text{haride alkoxide} \\
R-O^- Na^+ + MeI &\rightarrow R-O Me + NaI \\
\text{iodomethane methylated polysaccharide}
\end{align*}
\]

The carbamion is produced either in situ in the reaction vessel or separately and added to the reaction vessel, by reacting dry sodium hydride and dimethylsulphoxide, with heating (50°C). This methylation reaction can be performed on a relatively large scale or it can be employed in a modified form for the methylation of quantities from 1 to 20 mg.
If we consider a polysaccharide consisting only of hexose units, then, with an increasing degree of methylation, the absorption at 3600 cm\(^{-1}\) in the infrared spectrum of the methylated product, due to the presence of hydroxyl groups, will decrease until, in the infrared spectrum of the fully-methylated polysaccharide (theoretical methoxyl content = 45.6%), there will be no absorption band at 3600 cm\(^{-1}\), providing that all water and water vapour are excluded from the system.

Thus, the absence of an absorption band at 3600 cm\(^{-1}\) in the infrared spectrum of a methylated polysaccharide, consisting only of hexose units, and a methoxyl content of 45.6% would indicate complete methylation of the polysaccharide.

If the glucosidic linkages of the fully methylated polysaccharide are hydrolysed, the resulting partially-methylated monosaccharides are reduced, and the resulting \(\text{O-methyl-hexitols}\) are acetylated, the products, dissolved in a small quantity of a suitable solvent, can be introduced directly into a g.l.c. system where it can be separated into its components, identified, and passed directly into a mass spectrometer, where each component can be characterised.

Thus, a hexose unit in the original polysaccharide, will, by the above series of chemical reactions, produce a compound characteristic of the original hexose unit and the positions at which other hexose units were attached to it.

This is illustrated in figure II 2, using the example of a glucose unit in a polysaccharide chain, where the chain is continued through positions 1 and 6.
Fig. 11.2 The methylation analysis of a D-glucose unit in the polysaccharide chain.

D-glucose unit linked through positions 1 and 6

\[
\begin{align*}
\text{RO-CH}_2 & \quad \text{RO-CH}_2 \\
\text{O} & \quad \text{O} \\
\text{OH} & \quad \text{OH} \\
\text{OH} & \quad \text{OH} \\
\text{OR} & \quad \text{OR} \\
\end{align*}
\]

Methylation

\[
\begin{align*}
\text{RO-CH}_2 & \quad \text{RO-CH}_2 \\
\text{OMe} & \quad \text{OMe} \\
\text{OMe} & \quad \text{OMe} \\
\text{OMe} & \quad \text{OMe} \\
\end{align*}
\]

Hydrolysis

\[
\begin{align*}
\text{CH}_2\text{OH} & \quad \text{CH}_2\text{OH} \\
\text{OMe} & \quad \text{OMe} \\
\text{OMe} & \quad \text{OMe} \\
\text{OMe} & \quad \text{OMe} \\
\text{OH} & \quad \text{OH} \\
\end{align*}
\]

Reduction

\[
\begin{align*}
\text{CH}_2\text{OH} & \quad \text{CH}_2\text{OH} \\
\text{OMe} & \quad \text{OMe} \\
\text{OMe} & \quad \text{OMe} \\
\text{OMe} & \quad \text{OMe} \\
\end{align*}
\]

Acetylation

\[
\begin{align*}
\text{CH}_2\text{OAc} & \quad \text{CH}_2\text{OAc} \\
\text{OMe} & \quad \text{OMe} \\
\text{OMe} & \quad \text{OMe} \\
\text{OAc} & \quad \text{OAc} \\
\end{align*}
\]

G.L.C.-M.S.

1,5,6-tri-O-acetyl-2,3,4-tri-O-methyl-D-glucitol
There are two principal difficulties encountered when using g.l.c. as a separation technique for partially acetylated methyl-alditols. Firstly, difficulties are encountered in the separation of certain of the derivatives (1,2,5-tri-O-acetyl-3,4,6-tri-O-methyl-D-glucitol, \( T_{TMG} = 1.83 \) and 1,3,5-tri-O-acetyl-2,4,6-tri-O-methyl-D-glucitol, \( T_{TMG} = 1.82 \) [stationary phase 3% OV 225]). Improved separation of these two compounds can, however, be achieved by decreasing the temperature of the g.l.c. oven (Figure VII 1 (5)). At the reduced temperature, the compounds are retained longer on the g.l.c. column and the overlap of peaks is partially resolved.

Secondly, the series of chemical reactions described above produces products other than the alditol derivatives. These products arise principally from contaminants in the solvents, the reactants, and the atmosphere and they tend to give rise to large peaks on the g.l.c. traces. These products are characterised by temperature responses different from those of the alditol derivatives when subjected to g.l.c. and low ionisability under electron impact. Similar difficulties with so-called spurious peaks have been encountered by other workers in the field.

The relative areas under the peaks on the g.l.c. traces are proportional to the relative weights of the particular components, providing that all the compounds studied are chemically closely-related. Thus, a comparison of peak areas, after correcting for molecular weight, will yield the molar proportions of the various alditol derivatives.

A branched polyglucan will yield, on methylation analysis, assuming complete methylation, and total hydrolysis of all the glycosidic linkages, di-O-methyl-alditol acetate(s) and tetra-O-methyl-
alditol acetate(s) in the ratio 1:1. Lindberg et al, following earlier work by Chizhov et al, who first interpreted the behaviour of D-glucitol hexa-acetate under electron impact, studied a variety of acetylated partially methylated alditols and concluded:

1. Primary fragments are formed by fission between adjacent carbon atoms in the chain, the most preferred fission being between adjacent methoxylated carbon atoms (figure II 3).

Figure II 3 The primary preferred fragments formed on electron impact of TMG

\[
\begin{align*}
\text{CH}_2\text{OAc} \\
\text{H} \quad \text{C} \quad \text{O} \quad \text{Me} \\
\text{MeO} \quad \text{C} \quad \text{H} \\
\text{H} \quad \text{C} \quad \text{O} \quad \text{Me} \\
\text{H} \quad \text{C} \quad \text{OAc} \\
\text{CH}_2 \text{OMe}
\end{align*}
\]

2. The next favoured fission is between adjacent methoxylated and acetoxylated carbon atoms and the positive charge resides on the methoxylated carbon atom (figure II 4).
The primary non-preferred fragments formed on electron impact of TMG

3. Secondary fragments are formed from primary fragments by single or consecutive loss of acetic acid (m/e = 60), ketene (m/e = 42), methanol (m/e = 32) or formaldehyde (m/e = 30) (figure II 5).

The secondary fragments formed on electron impact of TMG

\[ \begin{align*}
205 & \xrightarrow{-(60)} 145 \\
161 & \xrightarrow{-(60)} 101 \xrightarrow{-(30)} 71 \\
161 & \xrightarrow{-(32)} 129 \xrightarrow{-(42)} 87 \\
117 & \xrightarrow{-(30)} 87
\end{align*} \]
4. The base peak of the spectrum (i.e. the largest peak) is generally m/e = 43 (CH$_3$CO$^+$).

5. Derivatives with the same substitution pattern give similar mass spectra typical of that substitution pattern (figure II 6).

Figure II 6 The fragmentation patterns of two isomeric acetylated partially methylated alditols

Thus, by the series of chemical reactions previously described, it is possible to produce from units within a polysaccharide structure, derivatives, characteristic of the original structural unit from which they were derived, which can be easily separated and characterised by the combined techniques of g.l.c. - m.s..

This procedure has thus greatly facilitated methylation analysis of the simpler polysaccharides.

Methylation of GS-5 B polysaccharide was achieved by a multi-step reaction based upon dimethylsulphinyl carbanion (VIA 7). The
infrared spectra of the products of each step of the methylation reaction are illustrated in figure VI 7 and it will be noted that the intensity of the band at 3600 cm\(^{-1}\) diminished with increasing methoxyl content.

The methoxyl contents of the products of the first methylation steps are illustrated in figure VI 6 along with the fate of the methylated product. As the methylation proceeded and the amount of material available for methoxyl determinations became limited, due largely to handling losses, determination of methoxyl contents was discontinued.

The conditions employed for hydrolysis of the methylated polysaccharide were chosen such that the glycosidic linkages in the methylated polysaccharide would be broken and any partially methylated fructose liberated would be destroyed.

The molar proportions of the various \(\beta\)-methyl-alditol derivatives, obtained by hydrolysing the polysaccharide for various lengths of time, are shown in table VI 7 and figure VI 8. The molar proportions of the di-\(\beta\)-methyl-alditol derivative and the tetra-\(\beta\)-methyl-alditol derivative are equal and constant (within experimental error) after 7.5 hours hydrolysis. A small increase in the molar proportion of the former and a small decrease in the molar proportion of the latter and that of the tri-\(\beta\)-methyl-alditol derivative are shown after hydrolysis for 24 hours.

The chromatogram of the 10 hour hydrolysis product is illustrated in figure VI 9 and the retention times of the components are shown in table VI 8. It will be observed that there are four major components.
It has been shown that GS-5 B polysaccharide consisted principally of glucose with a small amount of fructose (VIA 4) and that the latter would be largely destroyed on hydrolysis.

Thus, the four components are either glucitol derivatives or non-alditol derivatives.

Component 1 (peak 1) \( T_{\text{TMG}} = 1.00 \), was tentatively identified as 1,5-di-O-acetyl-2,3,4,6-tetra-O-methyl-\( \beta \)-glucitol (TMG) (\( T_{\text{TMG}} \) standard compound = 1.00, \( T_{\text{TMG}} \) literature\( ^{421} \) = 1.00).

Component 2 (peak 2) had a retention time similar to that expected for a tri-O-methyl-\( \beta \)-glucitol acetate but was later characterised as a non-alditol acetate derivative.

Component 3 (peak 3) \( T_{\text{TMG}} = 2.08 \) was tentatively identified as 1,5,6-tri-O-acetyl-2,3,4-tri-O-methyl-\( \beta \)-glucitol. (\( T_{\text{TMG}} \) standard compound = 2.02 and \( T_{\text{TMG}} \) literature\( ^{421} \) = 2.22).

Component 4 (peak 4) \( T_{\text{TMG}} = 3.76 \), was tentatively identified as a tetra-O-acetyl-di-O-methyl-\( \beta \)-glucitol, exact identification could not be achieved by g.l.c. alone. (\( T_{\text{TMG}} \) 1,3,5,6-tetra-O-acetyl-2,4-di-O-methyl-\( \beta \)-glucitol [standard compound] = 3.68. \( T_{\text{TMG}} \) 1,2,5,6-tetra-O-acetyl-3,4-di-O-methyl-\( \beta \)-glucitol [standard compound] = 3.78. \( T_{\text{TMG}} \) [literature\( ^{421} \)] = 4.21 and 4.26 respectively.)

The modification of the g.l.c. - m.s. interface (see Chapter VII) had the effect of reducing relative retention times of all components with absolute retention times greater than that of TMG. Some variation in the relative retention time of any one component, on different runs, was also noted.

This was overcome in later studies by using a separate g.l.c. system for the determination of the relative retention times.
Spectra of standard compounds obtained on our own apparatus were found to be similar to those reported by Lindberg et al.\(^8\) (see Chapter VII).

The mass spectra corresponding to the four peaks obtained on subjecting, acetylated, reduced, hydrolysed, methylated GS-5 B polysaccharide to g.l.c. are shown in figures VI 10–VI 13.

Spectrum 1 (corresponding to peak 1) contains the following fragments at intensities \(\geq 10\%\) of the base peak intensity.

\[ M/e = 43, 45, 71, 87, 101, 117, 129, 145, 161 \text{ and } 205. \]

These are the fragments obtained from \(1,5\)-di-O-acetyl-2,3,4,6-tetra-O-methyl-\(\beta\)-glucitol (TMG) on electron impact (figure II 3–II 5).

Thus with the combined evidence of g.l.c. – m.s. component 1 was identified as \(1,5\)-di-O-acetyl-2,3,4,6-tetra-O-methyl-\(\beta\)-glucitol and the origin of this compound is shown in figure II 7.

Component 1 must have originated from a non-reducing \(\beta\)-glucopyranosyl unit situated at a chain end.

Spectrum 2 (corresponding to peak 2) contains the following fragments at intensities \(\geq 10\%\) of the base peak intensity: \(m/e = 43\) and 149. There is also a distinctive triplet of peaks at an intensity slightly less than 10\% of the base peak intensity (\(m/e = 112, 113\) and 114). This component is not an alditol derivative, nor does it contain any detectable quantity of either \(1,3,5, -\text{tri-}O\)-acetyl-2\(\alpha\)-\(\text{tri-}O\)-methyl-\(\beta\)-glucitol or \(1,2,5, -\text{tri-}O\)-acetyl-3,4,6-\(\text{tri-}O\)-methyl-\(\beta\)-glucitol, both of which possess similar retention times.

Spectrum 3 (corresponding to peak 3) contains the following fragments at intensities \(10\%\) of the base peak intensity: \(m/e = 43, 87, 99, 101, 117, 129, 161\) and 189. These are the fragments obtained from \(1,5,6\)-tri-O-acetyl-2,3,4-tri-O-methyl-\(\beta\)-glucitol on electron impact (figure II 8).
Fig. 11.7 The origin of component 1 in the methylation analysis of GS-5 B polysaccharide.

1,5-di-O-acetyl-2,3,4,6-tetra-O-methyl-D-glucitol

2,3,4,6-tetra-O-methyl-D-glucitol

2,3,4,6-tetra-O-methyl-D-glucopyranose unit linked through position 1

2,3,4,6-tetra-O-methyl-D-glucopyranose unit linked only through position 1
Thus with the combined evidence of g.l.c. and m.s. component 3 was identified as 1,5,6-tri-O-acetyl-2,3,4-tri-O-methyl-D-glucitol and the origin of this component is illustrated in figure II 9.

Component 3 must have arisen from a D-glucopyranose unit linked through positions 1 and 6 and such a unit must have formed a linear portion of the polysaccharide structure. The presence of such units in relatively high proportions and the absence of other types of units linked only through two positions suggests that the GS-5 B polysaccharide has a backbone consisting principally of α-(1→6)-linked D-glucopyranose units.
Fig. 11.9 The origin of component 3 in the methylation analysis of GS-5 B polysaccharide

\[
\begin{align*}
\text{CH}_2\text{OH} & \quad \text{MeO} - \\
\text{CH}_2\text{OH} & \quad \text{-OMe} \\
\text{CH}_2\text{OH} & \quad \text{-OAc} \\
\text{CH}_2\text{OH} & \quad \text{CH}_2\text{OAc}
\end{align*}
\]

1,5,6-tri-O-acetyl-2,3,4-tri-O-methyl-D-glucitol

acetylation

\[
\begin{align*}
\text{MeO} - \\
\text{-OAc} \\
\text{-OAc} \\
\text{CH}_2\text{OAc}
\end{align*}
\]

2,3,4-tri-O-methyl-D-glucitol

reduction

\[
\begin{align*}
\text{CH}_2\text{OH} & \quad \text{MeO} - \\
\text{CH}_2\text{OH} & \quad \text{-OAc} \\
\text{CH}_2\text{OH} & \quad \text{-OAc}
\end{align*}
\]

2,3,4-tri-O-methyl-D-glucopyranose unit linked through positions 1 and 6

methylation

\[
\begin{align*}
\text{CH}_2\text{OH} & \quad \text{OMe} \\
\text{CH}_2\text{OH} & \quad \text{OMe} \\
\text{CH}_2\text{OH} & \quad \text{OMe}
\end{align*}
\]

2,3,4-tri-O-methyl-D-glucopyranose

hydrolysis

\[
\begin{align*}
\text{CH}_2\text{OH} & \quad \text{OMe} \\
\text{CH}_2\text{OH} & \quad \text{OMe} \\
\text{CH}_2\text{OH} & \quad \text{OMe}
\end{align*}
\]

D-glucopyranose unit linked through positions 1 and 6
Spectrum 4 (corresponding to peak 4) contains the following fragments at intensities $\geq 10\%$ of the base peak intensity: $m/e = 43, 87, 117, 129$ and 189. These are the fragments obtained from 1,3,5,6-tetra-$\alpha$-acetyl-2,4-di-$\alpha$-methyl-$\beta$-glucitol on electron impact (figure II 10).

Figure II 10 The fragments formed from 1,3,5,6-tetra-$\alpha$-acetyl-2,4-di-$\alpha$-methyl-$\beta$-glucitol on electron impact

Thus with the combined evidence of g.l.c. and m.s. component was identified as 1,3,5,6-tetra-$\alpha$-acetyl-2,4-di-$\alpha$-methyl-$\beta$-glucitol and the origin of this component is shown in figure II 11.

The molar proportions of the various alditol derivatives obtained from the methylation analysis of GS-5 B polysaccharide (determined as an average from 7.5, 8.5, 10 and 12 h hydrolysis products) are shown in table II 6.
The origin of component 4 in the methylation analysis of GS-5 B polysaccharide.

1,3,5,6-tetra-O-acetyl-2,4-di-O-methyl-\(\beta\)-glucitol

2,4-di-O-methyl-\(\beta\)-glucitol

2,4-di-O-methyl-\(\beta\)-glucopyranose unit linked through positions 1,3 and 6

\(\beta\)-glucopyranose unit linked through positions 1,3, and 6

Acetylation

Reduction

Hydrolysis

Methylation
Table II 6  The molar proportions of the products of the methylation analysis of GS-5 B polysaccharide

<table>
<thead>
<tr>
<th>Products of methylation analysis</th>
<th>Molar proportions</th>
<th>T&lt;sub&gt;TMG&lt;/sub&gt;</th>
<th>T&lt;sub&gt;TMG&lt;/sub&gt; standards</th>
</tr>
</thead>
<tbody>
<tr>
<td>1,5-di-O-acetyl-2,3,4,6-tetra-O-methyl-D-glucitol</td>
<td>17.65</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>1,5,6-tri-O-acetyl-2,3,4-tri-O-methyl-D-glucitol</td>
<td>65.74</td>
<td>2.08</td>
<td>2.02</td>
</tr>
<tr>
<td>1,3,56-tetra-O-acetyl-2,4-di-O-methyl-D-glucitol</td>
<td>16.59</td>
<td>3.76</td>
<td>3.68</td>
</tr>
</tbody>
</table>

Thus the proportion of di-O-: tri-O-: tetra-O-methyl-D-glucitol derivative is (within experimental error) 1:4:1.

The average repeating unit of GS-5 B polysaccharide as determined by methylation analysis is illustrated in figure II 12.

Figure II 12  The average repeating unit of GS-5 B polysaccharide
Investigation and characterisation of the primary linkages in GS-5 B polysaccharide by partial acid hydrolysis

Extensive use has been made, in previous work, of acid hydrolysis as a method of partially degrading polysaccharides. Experiments with disaccharides have established the order of stability of α-glucosidic linkages to acid hydrolysis, an α-(1→6) linkage being three or four times more resistant to hydrolysis than a secondary α-glucosidic linkage (figure II 13).

Partial hydrolysis of dextrans has been shown to yield products consisting almost exclusively of α-(1→6)-linked oligosaccharides (i.e. homologues of isomaltose). The high degree of polymerisation of some of the homologues suggests that this class of polysaccharide contains, as a general feature, a large number of contiguous α-(1→6)-linked β-glucopyranose units.

The GS-5 B polysaccharide was subjected to partial acid hydrolysis (VIA 12) and the hydrolysate and reduced hydrolysate were subjected to paper chromatography and electrophoresis.

It has been demonstrated that if the $R_M$ values (VA 7) of the members of an homologous series are plotted against their degree of polymerisation a straight line results. The $R_M$ value of each of the series of compounds obtained by the partial acid hydrolysis of GS-5 B polysaccharide (table VI 9) was plotted against its degree of polymerisation (either determined chemically [VD 7] or by comparison with standard compounds) (figure II 14).

The $R_M$ values of the series of compounds present in the reduced partial acid hydrolysate of GS-5 B polysaccharide were similarly plotted (figure II 15). In both cases a straight line was produced.
Fig. 11.13 Hydrolysis velocities of three α-glucobioses with 0.5M sulphuric acid at 85°C

extent of degradation %

- Kojibiose
- Nigerose
- Isomaltose

time (min)
Fig. 11.15  The partial acid hydrolysis of GS-5 B polysaccharide. Paper chromatography of the reduced product. Plot of $R_M$ versus degree of polymerisation

- - - - - chromatography solvent (a)
- - - - - staining reagent (a)
- - - - - chromatography solvent (b)
- - - - - staining reagent (a)
Fig. 11.16 The partial acid hydrolysis of GS-5 B polysaccharide. Paper electrophoresis. Plot of $1/M_s$ versus degree of polymerisation.

- Paper 1: Intercept = 1.0, gradient = 0.25
- Paper 2: Intercept = 0.99, gradient = 0.32

Electrophoresis buffer (a)
Staining reagent (a)
In addition, when the reduced partial hydrolysate of GS-5 B polysaccharide was subjected to molybdate electrophoresis, a plot of $\frac{1}{\bar{M}_n}$ (table VI 12 and VI 13) versus degree of polymerisation for each component in the reduced hydrolysate gave a straight line in accordance with the formula $\frac{1}{\bar{M}_n} = 1 + 0.31 n$ (where $n$ is the degree of polymerisation) characteristic of an homologous series (figure II 16).

The partial acid hydrolysate of GS-5 B polysaccharide was subjected to preparative paper chromatography in solvent (b) (VIA 12 (b)) and three components, compounds 1, 2 and 3, were eluted from the paper.

Compounds 1, 2 and 3 were purified by rechromatography in solvent (b), treated with activated charcoal, and evaporated to syrups.

The degree of polymerisation of each compound was determined (VD 7) and these are shown in table II 7. Crystalline derivatives of compounds 1 and 2 were prepared and their melting points and mixed melting points are tabulated in table II 7.

| Table II 7 Degree of polymerisation and properties of the crystalline derivatives of compounds 1, 2 and 3 obtained by the partial hydrolysis of GS-5 B polysaccharide |
|---------------------------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Compound | Degree of polymerisation | Crystalline derivative | M.p. of crystalline derivative | Melting point of mixed derivative | Literature Identity of compound derivative | Literature Identity of crystalline derivative |
| Compound 1 | 1 | β-penta-acetate | 130°C | 130°C | 132°C | glucose |
| Compound 2 | 1.8 | β-octa-acetate | 140°C | 140°C | 144°C | isomaltose |
| Compound 3 | 2.9 | | | | | |
Compound 1 had a degree of polymerisation equivalent to 1. The \( R_g \) value of compound 1 was similar to that of \( \alpha-D\)-glucose and the \( R_g \) value of reduced compound 1 to that of \( \alpha-D\)-glucitol. In addition, the crystalline pentaacetate had a melting point of 130°C and a mixed melting point (when compound 1 was mixed with an authentic sample of glucose \( \beta\)-pentaacetate) of 130°C. Thus compound 1 was identified as \( \alpha-D\)-glucose. This was substantiated by the findings of IIB 1 (a) in which glucose was identified in the total acid hydrolysate of GS-5 B polysaccharide by paper chromatography and IIB 2 in which GS-5 B polysaccharide was found to contain 93.4% glucose (by enzymic assay [VIA 4 (d)]).

Compound 2 had a degree of polymerisation of 2 (experimental value 1.8). The \( R_g \) value of compound 2 was similar to that of isomaltose and the \( R_g \) value of reduced compound 2 to that of isomaltitol. The crystalline octaacetate had a melting point of 140°C and a mixed melting point (when compound 2 is mixed with an authentic sample of isomaltose \( \beta\)-octaacetate) of 140°C. Thus, compound 2 was identified as isomaltose (\( \alpha-D\)-glucopyranosyl-(1\( \rightarrow \)6)-\( \alpha-D\)-glucose). This is in agreement with the results of IIB 5 which indicate that 83% of the \( \alpha-D\)-glucopyranosyl units in GS-5 B polysaccharide were linked through positions 1 and 6 or 1, 3 and 6.

Since the series of compounds in the partial acid hydrolysate form an homologous series, it follows that this series is based upon isomaltose, i.e. that the oligosaccharides contained \( \alpha-D\)-glucopyranosyl units linked through positions 1 and 6.

Compound 3 has a degree of polymerisation of 3 (experimental value 2.9). The \( R_g \) of compound 3 is similar to that of isomaltotriose and the \( R_g \) value of reduced compound 3 is similar to that of
isomaltotriitol. This is further evidence for the existence of an homologous series based on isomaltose.

The presence of such a series, containing homologues of high degrees of polymerisation, is indicative of a polysaccharide structure containing a large number of contiguous \( \alpha-(1 \rightarrow 6) \)-linked \( \alpha-D \)-glucopyranosyl units which form the polysaccharide backbone (i.e. a polysaccharide structure of the 'classic' dextran type).

IIB 7 Characterisation of the secondary glucosidic linkages in GS-5 B polysaccharide by partial acetylation

(a) The electrophoretic migrations of \( \alpha-D \)-glucobiobses and \( \alpha-D \)-glucobiitols in borate and molybdate buffers

The relationship between the structures of various sugars and their abilities to complex with borate and molybdate ions have been reviewed by Foster\(^ {428} \) and Weigel\(^ {429} \) respectively.

(i) Electrophoresis in sodium borate buffer, pH 10.0 (buffer (a)).

Boric acid acts as a Lewis acid accepting the electron pair of the base (e.g. \( \text{OH}^- \)) to form the stable anion \( \text{B(OH)}_4^- \). At alkaline pH this anion can form a weak negatively charged complex with many neutral sugars and their derivatives and such complexes will migrate under electrophoresis.

Foster considered that, in general, the magnitude of the net charge, and hence the rate of migration of the complex depends upon two factors.

The first, the number of contributions of the ring (either pyranose or furanose) and the open chain forms of the sugar (or its
derivative) which can interact with the borate ion and the second, the strength of the interaction at each complexing centre.

Reducing forms of the sugars, probably aldehyde forms, have been detected by polarographic studies\(^{430}\) and they have been shown to increase rapidly with increasing pH, and it would thus appear likely that these contribute significantly to the borate complex.

Angyal et al\(^{431}\) demonstrated, using n.m.r. spectroscopy, that borate ions form complexes with three syn-axial hydroxyl groups but not with an axial-equatorial-axial sequence.

Experiments with many sugars\(^{430}\) led to the conclusion that, for D-glucose, effective interactions of the borate ions with the ring and open chain forms of the sugar are limited to three possibilities (figure II 17), which are, in order of preference:

The interaction with the cis-hydroxyl groups at C-2 and C-4 in the acyclic form, structure (XXXIV).

The interaction with the equatorial hydroxyl groups at C-1 and C-2 in the pyranose or furanose ring form structure (XXXV).

The interaction with the hydroxyl groups at C-4 and C-6 in the acyclic form, structure (XXXVI).

Considering the α-glucobiose, both nigerose and isomaltose are able to form complexes of the type XXXIV and XXXV and hence the mobilities, in borate buffer, for these two compounds are relatively high (table II 8).

Maltose complexes with borate to produce only a complex of type (XXXV) and hence maltose has only a moderate mobility in borate buffer (table II 8). Kojibiose, too, only forms a single complex, of type (XXXVI) and hence possesses only a moderate mobility in borate.
**Fig. 11.17** Borate-D-glucose complexes — Possible structures

Legend

- carbon
- oxygen
- boron

(XXXIV)

(XXXV)
Fig. 11 17  (continued)

(XXXVI)
Table II 8  Electrophoresis in sodium borate buffer, pH 10.0

(buffer (a)) - Mobilities of the α-glucobiose

<table>
<thead>
<tr>
<th>Disaccharide</th>
<th>M&lt;sub&gt;g&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isomaltose  O-α-D-glucopyranosyl-(1→6)-D-glucose</td>
<td>0.69</td>
</tr>
<tr>
<td>Nigerose   O-α-D-glucopyranosyl-(1→3)-D-glucose</td>
<td>0.69</td>
</tr>
<tr>
<td>Maltose    O-α-D-glucopyranosyl-(1→4)-D-glucose</td>
<td>0.32</td>
</tr>
<tr>
<td>Kojibiose  O-α-D-glucopyranosyl-(1→2)-D-glucose</td>
<td>0.32</td>
</tr>
</tbody>
</table>

It is thus possible to differentiate between isomaltose or nigerose and maltose or kojibiose by electrophoresis in sodium borate buffer, pH 10.0.

(ii) Electrophoresis in sodium molybdate buffer, pH 5.0

In acid solution molybdates are able to form anionic complexes with certain polyhydroxy compounds<sup>432</sup> but the form of the complexing species was not initially apparent due to the formation of several isopolyacid ions at acid pH<sup>433</sup>.

Reduced α-D-glucose disaccharides were used to investigate the effects of substitution upon the complexing of the hexitol molecules and the molybdate ion<sup>434</sup>.

Analysis of the molybdate-glucobiitol complexes<sup>435</sup> indicated that they fall into three groups, which are as follows:

The 2 and 6 O-substituted glucitols (isomaltitol and kojibitol) form a complex in which the sugar alcohol: molybdenum atom ratio is 1:2.
The 4-O-substituted glucitols (maltitol) form a complex in which the sugar alcohol:molybdenum atom ratio is 1:1.

The 3-O-substituted glucitols (nigeritol) appear not to form stable complexes.

Potentiometric studies\(^{435}\) indicated that the complexing molybdate species was, in all cases, \(\text{Mo}_2\text{O}_7^{2-}\) and periodate oxidation studies of the essentially similar tungstate\(^{428}\) complexes indicated that in a complex, in which the substituted hexitol:molybdate atom ratio was 1:2 four adjacent hydroxyl groups were involved.

Thus for isomaltitol the complex would appear to be as shown in structure (XXXVII) and the kojibiitol:molybdate complex would appear to be of the type shown in structure (XXXVIII), both involving the complexing of the hydroxyl groups of four adjacent carbon atoms with a single \(\text{Mo}_2\text{O}_7^{2-}\) anion (i.e. a sugar alcohol:molybdenum ion ratio of 1:2).

The migration of maltitol is accounted for by the formation of a complex involving three adjacent hydroxyl groups of two molecules of maltitol and a single \(\text{Mo}_2\text{O}_7^{2-}\) anion (i.e. a sugar alcohol:molybdate atom ratio of 1:1).

The mobilities of complexes of types (XXXVII) and (XXXVIII) are high whereas complexes of type (XXXIX) have only a moderate mobility under the influence of an applied electric field. The mobilities for the four \(\alpha-D\)-glucobiitols are shown in table II 9.
Fig 11.18 Molybdate-D-glucitol complexes - Possible structures

Legend
- carbon
- oxygen
- molybdenum

(XXXVII)

(XXXVIII)
Fig. 11.18 (continued)
Table II 2  Electrophoresis in sodium molybdate buffer, pH 5.0 (buffer (b)) - Mobilities of the α-glucobiitols

<table>
<thead>
<tr>
<th>Disaccharide</th>
<th>M_g</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isomaltitol</td>
<td>0.70</td>
</tr>
<tr>
<td>Kojibiitol</td>
<td>0.70</td>
</tr>
<tr>
<td>Maltitol</td>
<td>0.43</td>
</tr>
<tr>
<td>Nigeritol</td>
<td>0.05</td>
</tr>
</tbody>
</table>

Using electrophoresis in sodium molybdate buffer, pH 5.0 (buffer (b)) it is possible to differentiate between isomaltitol or kojibiitol, maltitol and nigeritol.

(b) The acetolysis of GS-5 B polysaccharide

Matsuda et al. have demonstrated, through experiments with α-D-glucobioses, that the order of stability of α-glucosidic linkages to acetolysis reagents is α-(1→2) > α-(1→3) > α-(1→4) >> α(1→6) (figure II 19) which is the reverse of that found in partial acid hydrolysis (figure II 13).

This increased stability of the non-α-(1→6) linkages to acetolysis, as compared with acid hydrolysis, is thought to be due to a steric effect, the attacking species, the solvated acetylation ion, being able, more easily, to gain access at the more 'open' α-(1→6) linkages than at the more 'crowded' α-(1→2), α-(1→3) and α-(1→4) linkages.

Such a reversal of the rate of degradation permits the isolation of segments of the molecule containing non-α-(1→6) linkages.
in considerably larger yields than by acid hydrolysis and \( \alpha-(1\rightarrow2) \) \( ^{18} \),
\( \alpha-(1\rightarrow3) \) \( ^{18,40,76,78,93,102-107} \) and
\( \alpha-(1\rightarrow4) \) linkages have all been identified in various dextrans by this method.

In order to characterise the secondary linkages in GS-5 B polysaccharide a sample of the polysaccharide was subjected to partial acetolysis (VIA 13). After deacetylation, deionisation and evaporation the free sugars produced were subjected to paper chromatography in solvents (a) and (b). Staining reagents (a) and (c) were employed. The latter gives characteristic colours for the \( \alpha-D\)-glucobioses. Nigerose and isomaltose give a green-grey colouration, kojibiose gives a brown colouration and maltose a blue colouration.

The \( R_g \) values of the components of the acetolysate of GS-5 B polysaccharide, compared with the \( R_g \) values of the components of the partial hydrolysate of a standard dextran (Dextran T40 Sigma Biochemicals Ltd.) are shown in tables VI 14 and VI 15.

These results indicate the presence in the acetolysate of GS-5 B polysaccharide of a series of compounds having \( R_g \) values similar to those of the members of a homologous series based upon isomaltose.

In addition compounds, not members of the isomaltose homologous series and thought to contain secondary linkages derived from branch points in the polysaccharide structure, were present.

Compound 12, the compound, not a member of the isomaltose series, having the lowest degree of polymerisation was isolated from the partial acetolysate by preparative paper chromatography in
Fig. 11.19 Acetolysis of four α-glucobiose.
solvent (b). It was subjected to electrophoresis in buffer (a) and in its reduced form to electrophoresis in buffer (b). The mobilities of compound 12 and reduced compound 12 in buffers (a) and (b) respectively and the degree of polymerisation (Vd 7) of compound 12 are shown in Table II 10.

In addition, the properties of the crystalline derivative prepared from compound 12 are shown in Table II 11.

**Table II 10** The partial acetylation of GS-5 B polysaccharide -

<table>
<thead>
<tr>
<th>Some properties of compound 12</th>
</tr>
</thead>
<tbody>
<tr>
<td>Degree of polymerisation (Vd 7)</td>
</tr>
<tr>
<td>$R_g$ solvent (a)</td>
</tr>
<tr>
<td>$R_g$ solvent (b)</td>
</tr>
<tr>
<td>$M_g$ buffer (a)</td>
</tr>
<tr>
<td>$M_g$ of reduced compound 2 in buffer (b)</td>
</tr>
<tr>
<td>Colour with staining reagent (c)</td>
</tr>
</tbody>
</table>

**Table II 11** The partial acetylation of GS-5 B polysaccharide -

<table>
<thead>
<tr>
<th>Some properties of the crystalline derivative of compound 12</th>
</tr>
</thead>
<tbody>
<tr>
<td>Compound</td>
</tr>
<tr>
<td>-----------</td>
</tr>
<tr>
<td>Compound 12</td>
</tr>
</tbody>
</table>

Compound 12 had a degree of polymerisation of 2. The $R_g$ of compound 12 was similar to that reported for nigerose. The $M_g$ value when subjected to paper electrophoresis in buffer (a) was similar to that reported for nigerose and reduced compound 12 had a mobility of zero.
in buffer (b) as reported for nigeritol (see section IIB 7) (table II 10).

The crystalline β-octaacetate had a melting point similar to that reported for nigerose β-octaacetate. No material remained to perform a mixed melting point determination.

Thus, compound 12 was identified as nigerose [α-α-D-glucopyranosyl-(1→3)-D-glucose] and nigerose was the only 'secondary-linked' disaccharide to be detected in the partial acetolysate of GS-5 B polysaccharide. This is in agreement with the findings of section IIB 5 which show the presence of glucopyranosyl units linked through positions 1, 1 and 6 or 1,3 and 6 only.

Therefore, GS-5 B polysaccharide possesses a backbone of contiguous α-(1→6)-linked D-glucopyranosyl units with branches consisting of at least a single D-glucopyranosyl unit attached to the α-(1→6)-linked backbone by means of α-(1→3) glucosidic linkages.

IIB 8 The enzymic degradation of GS-5 B polysaccharide by P. lilacinum

dextranase

Dextranases [α-(1→6) glucan 6-glucanhydrolases EC 3.2.1.11] are enzymes that specifically hydrolyse the α-(1→6) linkages in dextrans.

Endodextranases i.e. dextranases that cleave dextrans in a random fashion, have been induced by growing certain strains of Penicillium on media containing dextran as the sole carbon source.

The enzymes produced by Penicillium lilacinum [strains I.M.I. 27830 (NRRL 895) and I.M.I. 79197 (NRRL 896)] and Penicillium
Funiculosum [strains I.M.I. 79195 (NRRL 1132) and I.M.I. 40235 (NRRL 1768)] have been investigated by Weigel et al.\textsuperscript{66,137,139}. These workers demonstrated that isomaltose was the major product of the enzymic hydrolysis of a branched dextran and that isomaltotriose and some glucose were produced along with limit dextrans. The limit dextrans all contained at least one secondary linkage and the careful characterisation of these limit dextrans enabled the mode of action of the fungal endodextranases to be determined. It was found that certain linkages within the structure of a branched dextran are resistant to attack by these enzymes.

These findings are summarised in figures II, 20 and II 21.

**Figure II 20** The mode of action of *P. lilacinum* dextrinase on a branched dextran

- **Legend to the following structures**
  - $\circ$ \textsuperscript{D} glucopyranose unit
  - $\rightarrow$ $\alpha-(1\rightarrow6)$ linkage
  - $\uparrow$ non-$\alpha-(1\rightarrow6)$ linkage
  - $\rightarrow\uparrow$ linkages resistant to enzymic hydrolysis
It will be noted that an $\alpha-(1\rightarrow6)$ linkage on the non-reducing side of the branch point is resistant to hydrolysis by *P. lilacinum* dextranase but is cleaved by the dextranase of *P. funiculosum*.

Thus, the smallest limit dextrin produced by the action of *P. lilacinum* dextranase on a branched dextran (e.g. one containing $\alpha-(1\rightarrow3)$ linkages) would be the pentasaccharide $O-\alpha-D$-glucopyranosyl-(1$\rightarrow$6)-[O-\alpha-D-glucopyranosyl-(1$\rightarrow$3)]$O-\alpha-D$-glucopyranosyl-(1$\rightarrow$6)-O-\alpha-D-glucopyranosyl-(1$\rightarrow$6)-D-glucose (XL).

The smallest limit dextrin produced by the action of *P. funiculosum* dextranase on a branched dextran (e.g. one containing $\alpha-(1\rightarrow3)$ linkages), would be the tetrasaccharide $O-\alpha-D$-glucopyranosyl-
The resistance of these linkages to attack by the fungal dextranases, however, is not total and incubation of the limit dextrins with the enzymes for extended periods will produce further hydrolysis. If the products of the hydrolysis by \textit{P. lilacinum} dextranase of a branched dextran (containing \(\alpha-(1\rightarrow 3)\) linkages as the sole secondary linkages) include the tetrasaccharide (XLI) it follows that the polysaccharide must contain a structure of the type represented by structure (XLII) in which \(\alpha-(1\rightarrow 3)\) linkages occur at non-branch points in the polysaccharide structure.
The endodextranase of \textit{P. lilacinum} was prepared as described in section VE 10. Qualitative digests were prepared, one containing GS-5 B polysaccharide, one containing the virtually unbranched \textit{S. bovis} strain I dextran and one, the reagent blank, containing enzyme and buffer but no polysaccharide (VIA 14).

After deionisation and concentration the hydrolysates were subjected to paper chromatography in solvent (a). The reagent blank contained no oligosaccharides. The chromatographic properties of some of the products of the hydrolysates are shown in tables VI 16 and VI 17* The degree of polymerisation of each component was estimated by comparison with standard compounds run on the same paper.

These results show that tetrasaccharide (XLI) is absent from the enzymic hydrolysate of GS-5 B polysaccharide indicating that all the secondary (\(\alpha-(1\rightarrow 3)\)) linkages in GS-5 B polysaccharide occur as branch points. This finding is in agreement with previous results (IIB 5) which show that 1,5,6-tri-\(\alpha\)-acetyl-2,3,4-tri-\(\beta\)-methyl-\(\alpha\)-glucitol is the only tri-\(\alpha\)-methyl-glucitol present in the acetylated, reduced, hydrolysed product of the methylation of GS-5 B polysaccharide.

A large scale enzymic hydrolysis of GS-5 B polysaccharide was prepared as described (VIA 14) and the product was subjected to preparative paper chromatography in solvent (c). The oligosaccharides having a degree of polymerisation \(> 3\) were eluted from the paper and purified by rechromatography in solvent (c). The yield of each component was determined, and the compounds were separately subjected to further paper chromatography in solvent (c).
The P. lilacinium dextranase digest of GS-5 B polysaccharide.

Preparative paper chromatography of the products of a large scale digest. Plot of $R_m$ versus degree of polymerisation.

\[ R_m = \log \left( \frac{1 - R_F}{R_F} \right) \]
The yield of each component and its $R_{\text{M}}$ value is shown in table VI 18. The $R_p$ value for each component was determined by extrapolation.

The $R_M$ value of each component was plotted against its estimated degree of polymerisation (figure II 22). Examination of figure II 22 shows that the oligosaccharides do not form a single homologous series. Compounds 24, 25 and 26 do appear to form a homologous series, the series being ascended by the addition of a single $\alpha-(\rightarrow 6)$-linked D-glucopyranose unit.

All the secondary linkages in GS-5 B polysaccharide occur at branch points, as shown by methylation studies (IIB 5) and by the absence of tetrasaccharide (XLI) from the P. lilacinum hydrolysate of GS-5 B polysaccharide. Considering the mode of action of the enzyme (figure II 20), the only possible structure of the pentasaccharide, compound 24, is as shown in structure (XL). Thus, component 24 contains only a single $\alpha-(\rightarrow 3)$ glucosidic linkage and as compounds 25 and 26 are members of the same homologous series (figure II 22) it follows that they must also contain a single $\alpha-(\rightarrow 3)$ linkage only.

The nonasaccharide, compound 28, in the P. lilacinum dextranase hydrolysate of GS-5 B polysaccharide must contain at least two secondary linkages since it is impossible to construct a nonasaccharide structure containing a single secondary linkage which does not also contain a hydrolysable isomaltosyl unit. Furthermore, compound 28, is not a member of the homologous series that contain compound 24, which possesses a single secondary linkage.

Of the possible structures of the nonasaccharide, compound 28, the one which has the maximum number of $\beta$-glucopyranose units between the two branch points is that shown in figure II 23 in which two...
D-glucopyranosyl units linked through positions 1, 3 and 6 are separated by two α-(1→6)-linked D-glucopyranosyl units.

Thus, in the GS-5 B polysaccharide there are a significant number of branches that are separated by two or less contiguous α-(1→6)-linked D-glucopyranose units. A quantitative P. lilacinum dextranase digest of GS-5 B polysaccharide was prepared as described (VIA 14 (b)) and samples were taken periodically and subjected to the Nelson test for reducing sugars (VD 3).

A standard dextran T 40 (a partially-degraded Leuconostoc mesenteroides NRRL B-512 dextran containing c.5% of α-(1→3) linkages) (Sigma Biochemicals Ltd.) was similarly hydrolysed for comparison purposes.

The results are shown in figure II 24. The results have been corrected for the carbohydrate content of the GS-5 B polysaccharide (table II 2).

It will be noted, by examination of figure II 24, that the enzymic degradation of GS-5 B polysaccharide is complete after 10 h. Since the quantitative digests were incubated at 37°C for 48 h, it follows that the enzymic degradation must have been complete.
Hence, the nonsaccharide, compound 28, (table VI 18) is a true limit dextrin and not the result of incomplete enzymic hydrolysis.

The extent of degradation of the polysaccharide (c 12%) (figure II 24) and the fact that the major product is isomaltose is indicative of a relatively complex polysaccharide structure.

If the branches each consisted of a single D-glucopyranosyl unit and if these branches occurred regularly on each fifth D-glucopyranosyl unit (figure II 12) it would not be possible to remove an isomaltosyl unit from the polysaccharide structure without cleaving a resistant linkage.

If however some branches are attached to D-glucopyranosyl units separated by less than four α(\(\rightarrow\) 6)-linked D-glucopyranosyl units, whilst other portions of the chain have sequences of five or more contiguous α-(\(\rightarrow\) 6)-linked D-glucopyranosyl units, it is possible that a distribution of products and a degree of degradation of GS-5 B polysaccharide, similar to that reported, will be obtained.

These findings are in agreement with the presence in the *P. lilacinum* dextranase digest of GS-5 B polysaccharide of a monosaccharide (see possible structure, figure II 23) which implies that some branches in GS-5 B polysaccharide are attached to D-glucopyranosyl units separated by two or less contiguous α-(\(\rightarrow\) 6)-linked D-glucopyranosyl units.

**IIB 9 Attempted fractionation and molecular weight determination of GS-5 B polysaccharide by gel permeation chromatography**

In gel permeation chromatography a column packed, usually with particles of a cross-linked dextran [Sephadex, Pharmacia (G.B.)] or a
Fig. 11.24: The P. lilacinum digest of GS 5 B polysaccharide. Quantitative digest

- **32.4%**
  - Standard dextran T40

- **12.3%**
  - GS-5 B polysaccharide

Reduction sugar as glucose/100µL solution

Time (hours)

100
similar material, is used to achieve separations between components of different molecular dimensions\textsuperscript{436}. The method has been used for desalting and exchanging the salt medium of colloids\textsuperscript{443}, and for separation and fractionation of mixtures of proteins, peptides, amino acids and dextrans\textsuperscript{437,438,442}.

The separation primarily depends upon the size of the molecules although adsorption phenomena do sometimes occur\textsuperscript{439}. The results that are most easily interpreted are those obtained with uncharged molecules\textsuperscript{440}, and oligosaccharides, derived from cellulose, which differ in size by only a single \(\text{D-glucopyranosyl} \) unit have been separated by gel permeation chromatography\textsuperscript{440}.

Fractionation of low molecular weight dextran\textsuperscript{441} and the separation of dextrans with weight average molecular weights ranging from \(\overline{M}_W = 3,400\) to \(\overline{M}_W = 41,000\)\textsuperscript{442} have been reported.

The molecular weight distribution of a polysaccharide can be illustrated by means of an elution diagram. As can be expected from the sieve mechanism, molecules are progressively more retarded with decreasing size, very large molecules being totally excluded from the gel.

By careful calibration of a gel column using standard neutral polysaccharides of known molecular weights, it is possible to produce an elution diagram in which the abscissa is a linear scale. Furthermore, the use of the partition coefficient, \(K\), of a particular fraction defined by the equation

\[
K = \frac{Ve - Vo}{Vt - Vo}
\]

where \(Ve = \) elution volume
\(Vo = \) void volume
\(Vt = \) bed volume

\((\text{VA 7 (a)})\)
eliminates the effects of gel bed compaction and column characteristics.

Gel columns of Sepharose 6B and Sepharose 2B (VA 6 (a)) and Sephadex G-200 and Sephadex G-100 (VA 6 (b)) were prepared as described, using Pharmacia K15/90 columns.

Experimental details of the procedures employed are given in section VIA 15.

Initial studies indicated that GS-5 B polysaccharide was poorly resolved on the Sepharose gels and was largely excluded from Sephadex G-100. Good resolution was however achieved on Sephadex G-200.

The Sephadex G-200 column was calibrated using a series of standard dextrans of known molecular weight. Using this information a plot of $K$, the partition coefficient between the liquid phase and the gel phase, versus the log $M_w$ of each dextran was constructed (figure VI 15).

The results obtained by subjecting GS-5 B polysaccharide to gel permeation chromatography on Sephadex G-200 are tabulated (table VI 19). From the elution volume of each fraction, the partition coefficient, $K$, was calculated. By reference to figure VI 15, the molecular weight of each of the fractions was determined and a plot of absorbance versus molecular weight was constructed (figure II 25).

The weight average molecular weight, $M_w$, of GS-5 B polysaccharide, calculated from figure II 25, is 49,600.

This is of the same order of magnitude as the molecular weights determined by Ceska et al. [89] for two glucans synthesised using purified glucosyltransferases from S. mutans OMZ 176. The molecular weights
of these two polysaccharides were found to be 24,000 and 70,000, by light scattering methods.

Inspection of figure II 25 shows that GS-5 B polysaccharide is polydispersed and a high concentration of material with a relatively low molecular weight ($\bar{M}_w = \text{approx. } 10,000$) can be noted.

This phenomena was further demonstrated by subjecting further samples of GS-5 B polysaccharide to gel permeation chromatography on Sephadex G-200 and by subjecting a sample of GS-5 B polysaccharide to ultracentrifugation on a Spinco Ultracentrifuge Model E (VIA 15 (c)). Again, an uneven molecular weight distribution was noted with a high concentration of material of low molecular weight. This is particularly apparent in the frame taken 28 minutes after the beginning of the run (figure II 26).

In order to investigate this apparent inhomogeneity, GS-5 B polysaccharide was subjected to preparative gel permeation chromatography (VIA 15 (d)). The elution diagram is shown in figure II 27.

Tubes 35 to 65 were combined (Fraction I) and Tubes 66 to 100 were combined (Fraction II).

The two fractions were freeze-dried, and dried in vacuo over phosphorus pentoxide. The yields were recorded and the fructose contents were determined (VD 5). The results are tabulated in table II 12.
Fig 11.25 The elution diagram of GS-5 B polysaccharide

D.M.S.O. treated polysaccharide

native GS-5 B polysaccharide

R_w = 49,000

molecular weight x 10^3

Absorbance

0.5
**Fig. 11.26 Ultracentrifugation of GS-5 B polysaccharide**

Speed = 59,780 rev/min.
Temperature = 20°C
Time elapsed from beginning of run, (t, min) is shown beneath individual frames
Fig. 11.27 Preparative gel permeation chromatography of GS-5 B polysaccharide on Sephadex G-200

Blue Dextran 2000

Glucose

GS-5B Polysaccharide

Fraction I

Fraction II

Absorbance

Elution volume (ml)

200

300

400

100

50
Table II 12  Preparative gel permeation chromatography of GS-5 B polysaccharide — yields and fructose contents of Fraction I and Fraction II

<table>
<thead>
<tr>
<th>Component</th>
<th>Yield mg from 500 mg of polysaccharide</th>
<th>Fructose content %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fraction I</td>
<td>250</td>
<td>2.89%</td>
</tr>
<tr>
<td>Fraction II</td>
<td>152</td>
<td>0.8%</td>
</tr>
</tbody>
</table>

If we consider that all the fructose present in the polysaccharide fractions is derived from sucrose units terminating dextran chains, we would expect the percentage of fructose in fraction II to be greater than that in fraction I.

Examination of table II 12 shows the fructose content of fraction I to be greater than that of fraction II. Thus all the fructose cannot be present in such structural units.

Furthermore, molecular weights determined from the information in table II 12, assuming all the fructose to be present in terminating sucrose units, are not in agreement with those obtained from gel permeation chromatography.

The low molecular weight material detected by gel permeation chromatography of GS-5 B polysaccharide could have three possible origins. It could represent a molecular species containing other than glucose units. Since it has been demonstrated that GS-5 B polysaccharide contains 2.5% of fructose, this material could be a low molecular weight fructan or fructose-containing polymer.

Conversely it could represent a glucan in which the D-glucopyranosyl units are linked differently from those in the bulk of GS-5 B polysaccharide.
Finally, this low molecular weight material may represent material essentially similar to that of the bulk of GS-5 B polysaccharide but possessing a lower molecular weight, this being produced by the mode of action of the synthesising enzyme.

The fructose contents of the two fractions (table II 12) shows fraction II to have a considerably lower fructose content than fraction I. This suggests that fraction II cannot be a fructose-containing polymer but that the fructose would appear to form part or the whole of a relatively high molecular weight polymer.

Hydrolysis of both fractions with the dextranase of P. lilacinum produces the same pattern of products (table VI 20) indicating that both fractions possess a branched structure.

Methylation analyses of fraction I and fraction II were performed as described (VIA 15 (d)). The g.l.c. traces obtained are illustrated in figures VI 16 and VI 17. The mass spectra of the alditol acetate peaks are shown in figures VI 18 to VI 23. A sample of glucose similarly treated was used to identify any spurious peaks arising from the reagents employed.

The molar proportions of the products of the methylation analysis are shown in table II 13.

Table II 13  The gel permeation chromatography of GS-5 B polysaccharide - The methylation of Fractions I and II - molar proportions of the acetylated methyl alditols

<table>
<thead>
<tr>
<th>Partially acetylated methyl alditol</th>
<th>Fraction I molar proportion</th>
<th>Fraction II molar proportion</th>
</tr>
</thead>
<tbody>
<tr>
<td>1,5-di-0-acetyl-2,3,4,6-tetra-0-methyl-D-glucitol*</td>
<td>16.9%</td>
<td>16.6%</td>
</tr>
<tr>
<td>1,5,6-tri-0-acetyl-2,3,4-tri-0-methyl-D-glucitol*</td>
<td>66.6%</td>
<td>66.7%</td>
</tr>
<tr>
<td>1,3,5,6-tetra-0-acetyl-2,4-di-0-methyl-D-glucitol*</td>
<td>16.4%</td>
<td>16.6%</td>
</tr>
</tbody>
</table>

* The fragmentation patterns of the partially acetylated methyl alditols and the origins of these compounds are illustrated in figures II 3 - II 5 and II 7 - II 11.
Examination of these results shows that fraction I is essentially similar to fraction II and both fractions have methylation products similar to those of GS-5 B polysaccharide.

Thus, it appears that the low molecular weight material observed on gel permeation chromatography and ultracentrifugation of GS-5 B polysaccharide is structurally similar to the bulk of GS-5 B polysaccharide and differs only in molecular size. It is presumed to be a product of the mode of action of the glucan-synthesising enzyme system of S. mutans GS-5.

Dimethylsulphoxide (d.m.s.o.) at elevated temperatures is reported to degrade dextran molecules. In order to investigate the possible degradation of GS-5 B polysaccharide under conditions similar to those used for the methylation procedure a sample of the polysaccharide treated with d.m.s.o. as described (VIA 15 (e)).

The elution diagram of the d.m.s.o.-treated GS-5 B polysaccharide is shown in figure II 25. Examination of figure II 25 illustrates that no appreciable decrease in molecular weight had occurred on treating GS-5 B polysaccharide with d.m.s.o.

IIB 10 Investigation of the polysaccharide structure in the region of the branch points by the mild acid hydrolysis of GS-5 B polysaccharide

Ullmann\textsuperscript{445} reported that, when starch was subjected to acid hydrolysis under very mild conditions, glucose, resulting from the breaking of terminal glucosidic linkages in the molecule, was the sole product.
Sidebotham et al. in an attempt to debranch *Leuconostoc mesenteroides* NRRL B-1299 S dextran, in order to render it open to attack by *P. lilacinum* dextranase, succeeded in producing structures containing \(\alpha-(\rightarrow 2)\)-linked D-glucopyranosyl units occurring at non-branch points in the polysaccharide structure (LXIII). The occurrence of such structural units was indicated by the presence of a tetrasaccharide (LXIV) in the *P. lilacinum* digest.

Further evidence for the above reaction was later provided by methylation studies on the mild hydrolysate.

From these studies it was suggested that *L. mesenteroides* NRRL B-1299 S dextran contains a structural segment possessing a D-glucose unit linked through positions 1, 2 and 6 whence the main chain is continued through positions 1 and 2 and the branch consists
of a D-glucopyranosyl unit (or units) attached through position 6 (LXV).

(LXV)

It was proposed to investigate the structure of GS-5 B polysaccharide in the region of the branch points by the application of a similar procedure.

It was noted (II B 1 (b)) that the products of the mild hydrolysis of GS-5 B polysaccharide under the optimum conditions for the production of fructose were fructose and glucose only. No oligosaccharides were detected in the hydrolysate (table VI 2).

Repetition of Experiment VIA 3, omitting the ethanol precipitation step gave fructose and glucose as the only low molecular weight products, indicating that oligosaccharides were not being produced in significant quantities and subsequently removed in the ethanol precipitation step.
Fig. 11.28 The mild acid hydrolysis of GS-5 B polysaccharide - glucose and reducing sugar released.
It was decided to conduct the experiment initially on a 25 mg sample of the polysaccharide for an unlimited length of time, monitoring the production of reducing sugars (VD 3) and glucose VD 4(a). The results are shown in figure II 28.

Examination of figure II 28 shows that glucose is released into the digest initially at a steady rate of approximately 0.025 mg per hour. After about 75 hours the rate of release of free glucose into solution begins to increase until a rate of 0.1 mg per hour is attained.

This could suggest that, in the initial stages, glucose is being released from the terminal positions of the dextran molecule but after about 75 hours more extensive hydrolysis of the polysaccharide occurs and more glucose is released.

Examination of the reducing sugars curve shows a broadly similar pattern. There is, however, an initial high release of reducing sugars followed by a fairly sharp fall. This feature is attributed to the release and degradation of fructose.

Release of reducing sugars then proceeds at a rate of 0.025 mg per hour until after 75 hours a more rapid release of reducing sugars is detected.

Examination, by paper chromatography in solvent (a) of the digest in the initial period shows fructose and glucose only to be present, whereas examination in the later stages shows the presence of oligosaccharides.

The experiment was repeated but was terminated after 75 hours. It was judged that after this period of time all the terminal glucose
units would have been removed but extensive degradation of the polysaccharide would not have occurred.

Paper chromatography in solvent (a) showed glucose and fructose (trace) to be the only low molecular weight species present.

Gel permeation chromatography of the mild hydrolysate on Sephadex G-200 indicated that the degraded polysaccharide had a molecular weight \( M_w = 29,000 \). (cf. original polysaccharide \( M_w = 49,600 \)).

Application of the methylation procedure with the subsequent hydrolysis, reduction and acetylation of the product (VIA 16 (b)) produced two partially acetylated methyl alditols.

The molar proportions of the two products are shown in table II 14 and the g.l.c. trace and mass spectra in figures VI 25 to VI 27.

<table>
<thead>
<tr>
<th>Partially acetylated methyl alditol</th>
<th>T-TMG</th>
<th>Molar proportion</th>
</tr>
</thead>
<tbody>
<tr>
<td>( 1,5\text{-di-O-acetyl-2,3,4,6-tetra-O-methyl-} ) ( \beta )-glucitol *</td>
<td>1.00</td>
<td>3.0%</td>
</tr>
<tr>
<td>( 1,5,6\text{-tri-O-acetyl-2,3,4-tri-O-methyl-} ) ( \beta )-glucitol *</td>
<td>2.10</td>
<td>97.0%</td>
</tr>
</tbody>
</table>

* The fragmentation patterns under electron impact and the origins of these partially acetylated methyl alditols are shown in figures II 3-5, II 7, II 8 and II 9.
The presence of the 2,3,4-tri-\(\alpha\)-methyl-D-glucitol derivative and the absence of any other tri-\(\alpha\)-methyl-D-glucitol derivative and any di-\(\alpha\)-methyl-D-glucitol derivatives from the product of the methylation analysis of the mild hydrolyzate of GS-5 B polysaccharide indicates that the remaining material must consist of a series of contiguous \(\alpha-(1\rightarrow 6)\)-linked D-glucopyranosyl units. Gel permeation chromatography of this remaining material (figure VI 24) shows it to have \(\bar{M}_w = 29,000\).

Thus the remaining material consists on average of structural units containing at least 180 contiguous \(\alpha\)-linked D-glucopyranosyl units (XLVI) occurring as an uninterrupted chain. Furthermore, the possibility of a structure of the type shown in partial structure (LXV) occurring in the native dextran can be excluded.

The yield of freeze-dried, partially-hydrolysed GS-5 B polysaccharide obtained from 25 mg of native polysaccharide was 24.7 mg. Examination of figure II 28 shows that 1.75 to 2.5 mg of the acid-degraded polysaccharide comprises low molecular weight material.

The very high yield of acid-degraded material can be accounted for by the higher moisture content of the freeze-dried product compared with that of the exhaustively-dried starting material and possible contamination by barium salts. A carbohydrate determination
on the acid-degraded material was not performed.

The results of the methylation analysis and molecular weight determinations of the acid-degraded GS-5 B polysaccharide suggest that a significant proportion of this material (approx. 60%) can be represented as in structure (XLVI).

IIB 11 The determination of the minimum length of the external branch chains in GS-5 B polysaccharide by swine kidney glucanhydrolase hydrolysis studies

Exodextranases are enzymes that cleave glucose units sequentially from terminal positions in dextran molecules leaving the molecular weight of the dextran essentially unaltered.

The isolation of such enzymes from Bacillus megatherium \(^{118}\), from Streptococcus mitis \(^{125-127,153,154}\) and from various animal tissues \(^{131-135,147,151}\) has been reported.

Zevenhuisen \(^{118}\) was able to show that two strains of Bacillus, obtained from the soil, were able to produce an exodextranase when grown in a medium containing clinical dextran as the sole carbon source. The enzyme was able to cleave several types of commercial dextran and a series of isomaltose homologues with the production of glucose.

Walker et al succeeded in isolating and purifying an exodextranase (\(\alpha-(1 \rightarrow 6)\) glucosidase) from a strain of Streptococcus mitis \(^{125-127,153,154}\). This enzyme was able to convert to glucose 0 – 27% of dextrans produced by various strains of S. bovis and L. mesenteroides.
Rosenfeld et al, noting that dextran injected intravenously into humans and animals underwent metabolic reactions, isolated from various animal tissues an enzyme capable of degrading a variety of dextrans.

The clinical dextrans Polyglucan, Polyglucin, Intradex and Macrodex were all degraded to extents ranging from approximately 14-35%.

Using such an enzyme system it is postulated that valuable information regarding the lengths of the branches in the streptococcal polysaccharides could be obtained, using enzymic methods, broadly similar to those employed by various workers for the elucidation of the fine structures of glycogen-type polysaccharides.

Samples of B. megatherium strain D2 were obtained from Dr. L.P.T.M. Zevenhuisen, Wageningen, the Netherlands. Samples of B. subtilis strain D5, on which the majority of Dr. Zevenhuisen's work had been performed were not available since the original cultures had been allowed to become inviable.

The organism was successfully recovered from the lyophilised state (VIA 17 (a)) and subcultured. Initial attempts to grow the organism in liquid culture was however unsuccessful (VIA 17 (b)). The recommended dextran-containing medium, medium M7, was an opaque medium (making culture growth very difficult to detect) containing tap water. The use of tap water, presumably added to supplement the mineral content of the medium, was, however, likely to inhibit bacterial growth. Activity indicated by an increase in reducing sugar content (VD 3) was not detected in the enzyme digest (VIA 17 (b)).
The method was substantially modified (VIA 17 (c)), the opaque medium, medium M7, being replaced by a clear medium, medium M8, based on basal medium for B. megatherium\(^447\). Cultures were grown in cotton-wool plugged Erle\'meyer flasks which were maintained at 30\(^\circ\)C with continual orbital shaking. The growth of the organisms was monitored (VB 1 (d)) and the cells were harvested during the period of exponential growth, before lysis of the bacterial cells could occur. The growth curve is illustrated in figure VI 28. The harvested cells were resuspended, in water, and ultrasonically disrupted. The resulting solution, after centrifugation, was incubated with S. bovis strain I dextran at 30\(^\circ\)C for 24 h and an increase in reducing sugar content was detected. A qualitative glucose oxidase/peroxidase test (VD 4 (c)), performed on the digest, was positive.

A single spot, migrating as glucose, was observed when the deionised, enzymic hydrolysate was subjected to paper chromatography in solvent (d). Microscopic examination of the stained (VB 4 [S1]) bacterial debris, after cell disruption, showed that many of the bacterial cells remained intact. In addition only 3 ml of enzyme solution was obtained and it was estimated that this would be insufficient for the prepared programme of work.

A further modification of the growth and extraction procedure was then adopted (VIA 17 (d)). The growth of the organisms was as described in VIA 17 (c). The cells were harvested and suspended in buffer B4 (0.16 g of bacterial cells (wet weight) per ml of buffer B4) and samples were subjected to ultrasonic disruption for various
lengths of time using both the micro- and medium probes. Ultrasonication using the former achieved little cell disruption when applied for up to nine minutes. Ultrasonication of the cells for fifteen minutes, using the medium probe, produced extensive cell disruption observed by visible microscopy.

The resulting solution, after removal of the debris by centrifugation at 3000 g was shown to have a protein content of 3.6 mg/ml (VD 6 (b)). When the solution was incubated at 30°C for 24 h with *S. bovis* Strain I dextran (VIA 17 (d)) an increase in reducing power of the digest (VD 3) was noted and the presence of glucose (VD 4 (c)) was also detected. Paper chromatography in solvent (d) of the deionised hydrolysate however showed the presence of a number of low molecular weight components.

A final modification of the growth and extraction procedure was then attempted (VIA 17 (e)). The *Bacillus* organism was initially subcultured through medium M8 to obtain a viable culture. A suspension of cells, collected by centrifugation, suspended in sterile water (2 ml) was employed as the inoculant. A growth curve was produced (figure VI 29) and the cells were harvested during the exponential period of growth.

The cells were ultrasonicated for a length of time sufficiently long to produce adequate cell disruption (determined microscopically). Undisrupted cells and large pieces of bacterial debris were removed from the suspension by centrifugation at 2000 g. Small fragments of bacterial debris were removed from the resulting solution by high speed centrifugation (25,000 g). The protein content of the solution prior to high speed centrifugation was
5 mg/ml and following high speed centrifugation was 2 mg/ml indicating that this procedure had removed a substantial amount of protein.

The resulting solution was incubated with *S. bovis* strain I dextran at 30°C for 24 h and an increase in reducing sugar (VD 3) and free glucose contents (VD 4 (c)) was noted. Paper chromatography of the deionised hydrolysate showed the presence of a number of low molecular weight products.

In this series of experiments it was shown to be possible to culture *B. megatherium* on a medium in which dextran was the only carbon source. However, the isolation of the exodextranase, proved difficult, and although a small quantity of the enzyme was obtained (VIA 17 (c)) subsequent increase in intensity of the ultrasonic bombardment appeared to break the cell organelles releasing a variety of general hydrolases into the solution.

Work was being conducted in our own laboratories to extract aminopeptidase from swine kidneys^44^ and an attempt was made to isolate glucanhydrolyase from the residue of this extraction procedure, by the method described by Rosenfeld^et al.131-135^ The enzyme preparation was purified by ammonium sulphate precipitation.

After three ammonium sulphate precipitations followed by concentration, a purified enzyme solution (protein content 7.8 mg/ml) (20 ml) was obtained.

The enzyme solution was incubated with acid-degraded *L. mesenteroides* NRRL B-512 dextran at 37°C for 50 h. A steady release of reducing sugars was shown over this period. T.l.c. of the enzymic hydrolysate showed glucose to be the only low molecular weight carbohydrate present (figure VI 31).
Gel permeation chromatography of the enzymic hydrolysate on a mixed-bed, Sephadex G-200/G-10 column indicated that the molecular weight of the dextran ($\overline{M}_w = 40,000$) was essentially unaltered by the action of the enzyme (figure VI 32). The enzyme was shown to be active between pH 3.0 and pH 6.8 with optimum activity at pH 3.8 - pH 4.5 (figure VI 33).

From swine kidney, kindly donated by Bowyers and Co. Ltd., London Road, Amersham, the glucanhydrolase was isolated by the scheme shown in figure VI 34.

The isolated enzyme preparation had a protein content of 7.0 mg/ml (VD 6 (b)) and a specific activity of $1 \times 10^3$ units/mg.

The enzyme was shown to be able to withstand freezing, exhibiting an activity loss of 15% on freeze-drying (figure VI 35). Gel permeation chromatography of a 75 h enzyme digest showed the molecular weight of the partially-hydrolysed substrate [acid degraded \textit{L. mesenteroides} NRRL B-512 dextran, (Dextran T 40)] to be essentially unaltered (figure VI 36) and after incubation for 15 days the presence of a low molecular weight product (glucose) could be detected.

Glucose was shown to be the only low molecular weight material present in the enzymic digest by t.l.c. The qualitative glucose oxidase/peroxidase test (VD 4 (c)) was positive.

The enzyme preparation was incubated for 500 h with substrates of \textit{S. mutans} GS-5 B polysaccharide and two native and one acid-degraded \textit{L. mesenteroides} dextrans, at 37°C. The results are shown in figure II 29.
Fig. 1129  The enzymic degradation of GS-5 B polysaccharide by the glucanhydrolase isolated from swine kidney

- 'S. mutans GS-5 B dextran
- Acid-degraded *L. mesenteroides* NRRL B512 dextran (Dextran T40)
- *L. mesenteroides* NRRL B612 dextran
- *L. mesenteroides* NRRL B-1375 (Birmingham) dextran

% conversion to glucose

Time (hours): 100, 200, 300, 400
The percentages of hydrolysis for these polysaccharides were:

- **S. mutans GS-5** dextran ... ... 15.5%
- acid-degraded **L. mesenteroides NRRL B-512** dextran (Dextran T 40) ... ... 8.5%
- native **L. mesenteroides NRRL B-512** dextran ... 8.5%
- native **L. mesenteroides NRRL B-1375** dextran (Birmingham) ... 9.0%

An external chain of a dextran can be defined as the series of D-glucopyranosyl units situated between a non-reducing chain end and a branch point.

The average external chain length of a polysaccharide can be obtained from the following formula:

\[ E = \frac{H}{B} + X \]

where

- **E** is the average external chain length
- **H** is the percentage of enzymic hydrolysis
- **B** is the percentage of branching
- **X** is the number of units in the external chain not removed by the enzyme.
Figure II 30  Possible enzymic degradations of dextrans in the region of branch points by swine kidney glucanhydrolase

![Diagram of enzymic degradations of dextrans](image)

\[(\text{XLVII})\]

\[(\text{XLVIII})\]

In the examples shown in figure II 30, where structures (XLVII) and (XLVIII) are both considered to represent the average repeating unit of the polysaccharide, \( H = B = 20\% \) and \( x = 1.0 \) and 0.

Thus, the number of units in the external chain would be 2 and 1 respectively.

Considering structure (XLVII), the \( \beta \)-glucopyranose unit linked \( \alpha-(1\rightarrow3) \) to the main \( \alpha-(1\rightarrow6) \)-linked \( \beta \)-glucopyranose chain will not be removed by the swine kidney glucanhydrolase. Thus \( x \gg 1 \).
Assuming that the branches in the polysaccharides studied are of this type (mild acid hydrolysis studies on GS-5 B polysaccharide shows that a structure of type (XLVIII) is absent) the minimum average external chain length, E, is given by the expression

\[ E = \frac{H}{B} + 1 \]

The percentage of degradation obtained in the present studies are significantly lower than some of the results reported by Rosenfeld et al.\textsuperscript{133,143} and Walker et al.\textsuperscript{153,154}.

That this phenomenon was not due to enzyme deactivation was demonstrated by the addition of further glucanhydrolyase to a 500 h digest (substrate Dextran T 40). No further release of reducing sugar was detected. The addition of further substrate (Dextran T 40) to a 500 h digest however produced a further steady release of reducing sugar, showing that the glucose produced was not inhibiting the enzyme. Thus, the lower degrees of hydrolysis must arise as the result of differences in the enzyme preparation used, the conditions employed or the polysaccharides investigated.

It is recognised, however, that the swine kidney glucanhydrolyase preparation used in the present studies represents a complex mixture of enzymes and the results obtained should be treated accordingly.

The percentage hydrolysis obtained when \textit{S. mutans} GS-5 B polysaccharide was incubated with swine kidney glucanhydrolyase indicates that at least 15.5% of the D-glucopyranosyl units in GS-5 B polysaccharide are located in the external chain. The degree
of branching of GS-5 B polysaccharide is 17%, determined by methylation analysis (IIB 5). Thus the minimum average external chain length of GS-5 B polysaccharide is approximately 2 and the average repeating unit can be represented as in figure II 31.

Figure II 31 Possible average repeating unit for GS-5 B polysaccharide

The absence of a structure of the type shown in partial structure XLIX was demonstrated by mild acid hydrolysis (IIB 10).
IIC The GS-5 A Polysaccharide

The lesser-soluble fraction of the polysaccharide elaborated by *S. mutans* GS-5 was cultured and extracted as described in VE 2 and VE 3. Two separate batches of GS-5 A polysaccharide were produced and unless, otherwise stated, the work described in this section has been performed on GS-5 A polysaccharide, Batch 1.

Examination of table II 1 will show that while the yields of GS-5 B polysaccharide per 100 g of sucrose appear similar for both batches, those of GS-5 A polysaccharide differ markedly between the batches. This difference could arise due to variations in the culture and extraction conditions between batches.

Both batches of GS-5 A polysaccharide were investigated.

IIC 1 Determination of the monosaccharide components of GS-5 A polysaccharide by acid hydrolysis

(a) Hydrolysis at the optimum conditions for the production of glucose

GS-5 A polysaccharide was hydrolysed as described (VIA 19) and the chromatographic properties of the hydrolysis products are shown (table VI 23). The results show the presence of a single spot migrating as glucose when the hydrolysate was subjected to paper chromatography and borate electrophoresis, and as glucitol when the reduced hydrolysate was subjected to molybdate electrophoresis.
(b) **Hydrolysis at the optimum conditions for the production of fructose**

GS-5 A polysaccharide was hydrolysed as described (VIA 20) and the chromatographic properties of the hydrolysis products are shown in table VI 24.

The results show the presence of two monosaccharide components migrating as fructose and glucose when the hydrolysate was subjected to paper chromatography.

Two spots corresponding to glucitol and mannitol were observed when the reduced hydrolysate was subjected to borate electrophoresis. The relatively high intensity of the fructose spot suggests that GS-5 A polysaccharide contains a relatively high percentage of fructose compared with GS-5 B polysaccharide.

**II.C.2 Composition**

Quantitative estimation of monosaccharides, ash, nitrogen, protein and carbohydrate contents were determined as described (VIA 21). The results are tabulated in table II 15.

**Table II 15 The Composition of GS-5 A polysaccharide**

<table>
<thead>
<tr>
<th>Carbohydrate content %</th>
<th>Glucose content %</th>
<th>Fructose content %</th>
<th>Protein content %</th>
<th>Ash content %</th>
<th>Specific rotation $[\alpha]_D^{25}$ c 0.29, M NaOH</th>
<th>$[\alpha]_D^{25}$ c 0.164, M NaOH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Batch 1</td>
<td>70.8</td>
<td>28.7</td>
<td>43.7</td>
<td>14.57</td>
<td>12.47 +41.3°</td>
<td></td>
</tr>
<tr>
<td>Batch 2</td>
<td>74.0</td>
<td>47</td>
<td>29</td>
<td>24.9</td>
<td>3.25 +128°</td>
<td></td>
</tr>
</tbody>
</table>
These results show that in both batches approximately 70% of the material is carbohydrate, and that glucose and fructose are the sole monosaccharide components. The proportion of glucose and fructose in the GS-5 A polysaccharide varies between the batches.

It would appear most likely that *S. mutans* GS-5 is able to elaborate from sucrose two (at least) separate polysaccharides, one a polyfructan and the other a polyglucan and the proportion of the two polysaccharides varies significantly with slight variations in the culture or extraction procedures.

**II C 3 Types and Percentages of the variously linked D-glucose residues in GS-5 A polysaccharide as determined by methylation analysis.**

GS-5 A polysaccharide was subjected to methylation analysis as described (VIA 22). The g.l.c. trace of the acetylated, reduced, hydrolysed, methylated GS-5 A polysaccharide, Batch I, is shown in figure VI 38 and the relevant mass spectra are illustrated in figure VI 39 - VI 42. The retention times and the molar proportions of each component are shown in table II 16.

The infrared spectrum of methylated GS-5 A polysaccharide shows no absorption in the -OH absorption region, indicative of complete methylation (figure VI 37).
Table II 16  The methylation analysis of GS-5 A polysaccharide —
molar proportions of the products

<table>
<thead>
<tr>
<th>Partially acetylated methylalditol</th>
<th>Molar proportion %</th>
<th>T^TMG</th>
<th>T^TMG standards</th>
</tr>
</thead>
<tbody>
<tr>
<td>1,5-di-O-acetyl-2,3,4,6-tetra-O-methyl-D-glucitol</td>
<td>20.5</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>1,3,5-tri-O-acetyl-2,4,6-tri-O-methyl-D-glucitol</td>
<td>1.6</td>
<td>1.80</td>
<td>1.90</td>
</tr>
<tr>
<td>1,5,6-tri-O-acetyl-2,3,4-tri-O-methyl-D-glucitol</td>
<td>58.4</td>
<td>2.20</td>
<td>2.20</td>
</tr>
<tr>
<td>1,3,5,6-tetra-O-acetyl-2,4,di-O-methyl-D-glucitol</td>
<td>19.4</td>
<td>4.30</td>
<td>4.20</td>
</tr>
</tbody>
</table>

In Table II 17 the retention times and molar proportions of the
components of the methylation analysis of GS-5 A polysaccharide,
batch 2, are shown. The g.l.c. trace and the mass spectra, which are
not illustrated, were similar to those obtained for Batch 1.

Table II 17  The methylation analysis of GS-5 A polysaccharide — the
molar proportions of the methylation products

<table>
<thead>
<tr>
<th>Partially acetylated methyl alditol</th>
<th>Molar proportion %</th>
<th>T^TMG</th>
<th>T^TMG standards</th>
</tr>
</thead>
<tbody>
<tr>
<td>1,5-di-O-acetyl-2,3,4,6-tetra-O-methyl-D-glucitol</td>
<td>20.8</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>1,3,5-tri-O-acetyl-2,4,6-tri-O-methyl-D-glucitol</td>
<td>3.9</td>
<td>1.80</td>
<td>1.90</td>
</tr>
<tr>
<td>1,5,6-tri-O-acetyl-2,3,4-tri-O-methyl-D-glucitol</td>
<td>55.9</td>
<td>2.16</td>
<td>2.20</td>
</tr>
<tr>
<td>1,4,5,6-tetra-O-methyl-2,4-di-O-methyl-D-glucitol</td>
<td>19.4</td>
<td>4.00</td>
<td>4.20</td>
</tr>
</tbody>
</table>
The fragmentation patterns under electron impact and the origins of these partially acetylated methyl alditols are shown in figures II 3-5 and II 7 to II 11, II 32 and II 33. Considering Batch 1 the molar proportion of the di-O: tri-O: tetra-O-methyl-D-glucitol derivatives is (within experimental error) 1:3:1. and approximately 3% of the tri-O-methyl-D-glucitol derivatives consists of 1,3,5-tri-O-acetyl-2,4,6-tri-O-methyl-D-glucitol.

Neglecting temporarily the 1,3,5-tri-O-acetyl-2,4,6-tri-O-methyl-D-glucitol, the average repeating unit of CS-5 A polysaccharide can be represented as in figure II 34.

**Figure II 32** The fragments formed from 1,3,5-tri-O-acetyl-2,4,6-tri-O-methyl-D-glucitol on electron impact

\[
\begin{align*}
\text{C} & \quad \text{H}_2 \text{OAc} \\
\text{H} & \quad \text{C} - \text{OMe} \\
\text{AcO} & \quad \text{C} \quad \text{H} \\
\text{H} & \quad \text{C} - \text{OMe} \\
\text{H} & \quad \text{C} - \text{OAc} \\
\text{CH}_2\text{OMe} & \quad \\
\end{align*}
\]
Fig. 11 33 The origin of component 3 in the methylation analysis of CS-5 B polysaccharide

\[
\begin{align*}
&\text{CH}_2\text{OAc} \\
&\text{HO} \\
&\text{OMe} \\
&\text{OMe} \\
&\text{OH} \\
&\text{CH}_2\text{OMe}
\end{align*}
\]

1,3,5-tri-O-acetyl-2,4,6-tri-O-methyl-D-glucitol

\[
\begin{align*}
&\text{CH}_2\text{OH} \\
&\text{HO} \\
&\text{OMe} \\
&\text{OMe} \\
&\text{OH} \\
&\text{CH}_2\text{OMe}
\end{align*}
\]

2,4,6-tri-O-methyl-D-glucitol

\[
\begin{align*}
&\text{CH}_2\text{OMe} \\
&\text{HO} \\
&\text{OMe} \\
&\text{OMe} \\
&\text{OH} \\
&\text{CH}_2\text{OMe}
\end{align*}
\]

2,4,6-tri-O-methyl-D-glucopyranose unit linked through positions 1 and 3

\[
\begin{align*}
&\text{CH}_2\text{OMe} \\
&\text{OR} \\
&\text{OMe} \\
&\text{OMe} \\
&\text{OR}
\end{align*}
\]

2,4,6-tri-O-methyl-D-glucopyranose

\[
\begin{align*}
&\text{CH}_2\text{OH} \\
&\text{OR} \\
&\text{OR} \\
&\text{OR}
\end{align*}
\]

D-glucopyranose unit linked through positions 1 and 3
Thus it would appear that GS-5 A polysaccharide is more highly branched than GS-5 B polysaccharide. The presence of the 1,3,5-tri-O-acetyl-2,4,6-tri-O-methyl-D-glucitol could be due to α-(1→3)-linked D-glucopyranosyl units occurring at non-branch points in a principally α-(1→6)-linked D-glucopyranosyl chain or to the presence of a separate 'mutan'-type polysaccharide. This latter case would arise if the *Streptococcus mutans* organism elaborates simultaneously a dextran and a 'mutan'-type polysaccharide. The second batch of polysaccharide, Batch 2, is structurally similar to Batch 1. The most significant difference being the slightly higher proportion of 1,3,5-tri-O-acetyl-2,4,6-tri-O-methyl-D-glucitol in the methylation analysis product of Batch 2.

The enzymic degradation of GS-5 B polysaccharide by

\[ \text{P. lilacinum dextranase} \]

GS-5 A polysaccharide was enzymically degraded as described (VIA 23). The quantitative digest showed hydrolysis to be complete after a period of 5 hours incubation. Paper chromatography of a 48 h digest showed that a 'branch' tetrasaccharide was absent from the hydrolysate (table VI 25).
It is possible that the tetrasaccharide could have been produced in quantities so small as to be undetectable. Conversely the α-(1→3) linkages which occur at non-branch points in the polysaccharide could occur in close proximity to each other or to branch points, or they may form part of an essentially α-(1→3) linked polyglucan.

IIC 5 The determination of the minimum length of the external branch chains in GS-5 A polysaccharide by swine kidney glucanhydrolyase hydrolysis studies

A quantitative swine kidney glucanhydrolyase digest of GS-5 A polysaccharide was prepared and analysed as described (VIA 24). The results are shown in figure II 35.

Examination of figure II 35 shows a degree of hydrolysis of GS-5 A polysaccharide by swine kidney glucanhydrolyase of 8%, corresponding to 20% degradation of the glucose-containing portion of the polysaccharide (assuming the fructose-containing portion to be undegraded).

It has been demonstrated that the degree of branching of GS-5 A polysaccharide is 20% (IIC 3). Thus, the minimum average external chain length of GS-5 A polysaccharide is 2 and the average repeating unit of the polysaccharide can be represented by partial structures (L) and (LI) in figure II 36. If a structural unit of type (LI) is present in GS-5 A polysaccharide (such a unit was shown to be absent in GS-5 B polysaccharide) then in the equation \( E = \frac{H}{B} + X \) (page 131) \( X = 0 \), and the minimum average external chain length corresponding to 20% degradation by the swine kidney glucanhydrolyase = 1.
Fig. 11. The enzymic degradation of GS-5 B polysaccharide by the glucanhydrolase isolated from swine kidney.

% conversion to glucose

S. mutans GS-5 B dextran
S. mutans GS-5 A dextran
acid-degraded L. mesenteroides NRRL B-512 dextran (Dextran T 40)

time (hours)
Figure II 36  Possible average repeating units of GS-5 A
polysaccharide (neglecting α-(1→3)-linkages which
do not form branch points)

(L)

(LI)
Conclusions

Streptococcus mutans GS-5 was shown to elaborate, in vitro, when incubated on a sucrose-based medium, a large amount of extracellular polysaccharide (approximately 1 g per 100 g of sucrose) which was fractionated, by ethanol precipitation, into a soluble fraction, GS-5 B polysaccharide, and a lesser-soluble fraction, GS-5 A polysaccharide.

The soluble fraction was shown to be essentially a dextran-type polysaccharide (a polyglucan in which the majority of the anhydroglucose units are linked $\alpha-(1\rightarrow 6)$).

Approximately $17\%$ of the anhydroglucose units were linked through secondary positions, and all the secondary linkages were shown to be $\alpha-(1\rightarrow 3)$ linkages and all occurred at branch points. $17\%$ of the anhydroglucose units were shown to be located at terminal, non-reducing positions.

Mild acid hydrolysis followed by methylation analysis of the resulting partially hydrolysed polysaccharide, indicated that a large proportion of the molecule comprised an uninterrupted series of contiguous $\alpha-(1\rightarrow 6)$-linked D-glucopyranose units.

Swine kidney glucanhydrolase degradation studies suggested that the minimum average external chain length of the polysaccharide was relatively short (2) compared to the average external chain length reported for amylopectin (15-16)\(^5\).

Considering the three types of structure originally proposed for amylopectin, namely the laminated structure (Haworth) (LII), the herring bone or comb-like structure (Staudinger) (LIII) and the
ramified or tree-like structure (Meyer) (LIV) (Figure II 37), the evidence obtained from the mild acid hydrolysis experiment and the swine kidney glucanhydrolase experiment indicates a structure for GS-5 B polysaccharide tending toward the Staudinger model (LIHI).

The possibility of the presence of a low percentage of relatively long branches (B chains) bearing shorter branches (A chains) is not however excluded.

GS-5 B polysaccharide was shown to be polydispersed $M_n = 49,000$, and a low molecular weight component, structurally similar to the bulk of GS-5 B polysaccharide and arising, presumably, from the mode of action of the synthesising enzyme, was shown to be present.

The lesser-soluble fraction, GS-5 A polysaccharide, appeared more complex. It contained a large percentage of fructose, and the proportion of fructose to glucose varied between the two batches of polysaccharide prepared.

Methylation analysis showed that the glucan contained approximately 20% of secondary, $(\alpha \rightarrow 3)$, linkages occurring at branch points and approximately 20% of the anhydroglucose units were located at non-reducing terminal positions.

The presence of 1.6 to 3.9% of $\alpha-(\alpha \rightarrow 3)$-linked $D$-glucopyranosyl units occurring at non-branch points was shown by methylation analysis.

The $P. lilacinum$ dextranase digest of GS-5 A polysaccharide did not contain any tetrasaccharide. This suggests that the $\alpha-(\alpha \rightarrow 3)$-linked $D$-glucopyranosyl units that do not occur at branch points occur:
Fig. 11 37 Possible structural models for GS-5 B polysaccharide

Legend

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>R</td>
<td>reducing group</td>
</tr>
<tr>
<td>→</td>
<td>secondary linkage occurring at a branch point</td>
</tr>
<tr>
<td>A</td>
<td>chains linked solely through their reducing groups to the rest of the molecule</td>
</tr>
<tr>
<td>B</td>
<td>chains to which A chains are attached but which themselves are linked through their reducing groups to another chain</td>
</tr>
<tr>
<td>C</td>
<td>chains to which other chains are attached and which carry reducing groups</td>
</tr>
</tbody>
</table>
a) as part of a separate, essentially non-dextran-type polysaccharide, and so are not removed by the endodextranase.

b) as contiguously-linked \(\alpha-(1\rightarrow 3)\)-linked D-glucopyranosyl units within a dextran-type structure.

c) in close proximity to branch points.

From swine kidney glucanhydrolase degradation studies, an average minimum external chain length of 2 was obtained, which was similar to that obtained for GS-5 B polysaccharide.

Comparison of the soluble and lesser-soluble polyglucans elaborated by \textit{S. mutans} GS-5 show them to be similar types of polysaccharide. The lesser-soluble fraction possesses a higher degree of branching and has a small percentage of secondary linkages which occur at non-branch points.

The fructose-containing portion of GS-5A polysaccharide was not fully investigated. However, it will be noted (Chapter IV) that \textit{Streptococcus salivarius} strain S1 levan is rapidly degraded by the swine kidney glucanhydrolase but that such a rapid degradation of the fructose-containing portion of GS-5 A polysaccharide does not occur. Furthermore high molecular weight levens \((M_w 25 \times 10^6)\) are reported to be soluble\(^{193,456}\). Thus, the fructose-containing portion of GS-5 B polysaccharide is not typical of the bacterial levens so far investigated, and the possibility exists that this material is chemically or physically bound to the rest of the GS-5 A polysaccharide.
Chapter III  THE POLYSACCHARIDE ELABORATED BY STREPTOCOCCUS MUTANS

OMZ 51

IIIA  Preparation and extraction

The organism Streptococcus mutans OMZ 51 was kindly donated by Dr. W.H. Bowen of the Royal College of Surgeons of England.

Two polysaccharide fractions OMZ 51 A polysaccharide (precipitated with 40% ethanol) and OMZ 51 B polysaccharide (precipitated with 70% ethanol) (VIB 1) were obtained. The yields of the polysaccharide fractions are shown in table III 1.

Table III 1  The yields of the polysaccharide fractions produced by S. mutans OMZ 51

<table>
<thead>
<tr>
<th>Yield of fraction A</th>
<th>Yield of fraction B</th>
</tr>
</thead>
<tbody>
<tr>
<td>g from 100 g of sucrose</td>
<td>g from 100 g of sucrose</td>
</tr>
<tr>
<td>0.68</td>
<td>1.03</td>
</tr>
</tbody>
</table>
III B The OMZ 51 B polysaccharide

III B 1 Determination of the monosaccharide components by acid hydrolysis

A small quantity of the polysaccharide was hydrolysed under conditions designed to completely cleave glucosidic linkages in the polysaccharide with the minimum degradation of the glucose released (VIB 2). The chromatographic properties of the product of hydrolysis are shown in table VI 26. The hydrolysate when subjected to paper chromatography in solvent (d) gave only a single spot migrating as glucose.

A sample of OMZ 51 B polysaccharide was hydrolysed under mild conditions designed to cleave fructosidic linkages. Two spots migrating as glucose and fructose were detected when the hydrolysate was subjected to paper chromatography in solvent (d) (table VI 27).

III B 2 Composition

Quantitative estimations of the carbohydrate and non-carbohydrate A components of OMZ 51 B polysaccharide were conducted (VIB 4). The results are shown in table III 2.

Table III 2 Composition of OMZ 51 B polysaccharide

<table>
<thead>
<tr>
<th>Carbo-</th>
<th>Glucose</th>
<th>Fructose</th>
<th>Protein</th>
<th>Ash</th>
<th>Specific rotation</th>
</tr>
</thead>
<tbody>
<tr>
<td>hydrate</td>
<td>content</td>
<td>content</td>
<td>content</td>
<td>content</td>
<td>[α]D^25 c 1.97, M NaOH</td>
</tr>
<tr>
<td>%</td>
<td>%</td>
<td>%</td>
<td>%</td>
<td>%</td>
<td></td>
</tr>
<tr>
<td>98.8</td>
<td>94.36</td>
<td>6.0</td>
<td>1.2</td>
<td>0</td>
<td>+127°</td>
</tr>
</tbody>
</table>
IIIB 3 Types and percentages of the variously linked D-glucose residues in OMZ 51B polysaccharide as determined by methylation analysis

The methylation analysis of OMZ 51 B polysaccharide was performed as described (VIB 5). The relevant g.l.c. trace and mass spectra are shown in figures VI 44 – VI 47 and the infrared spectrum of the methylated polysaccharide is shown in figure VI 48. The absence of an absorption band in the region 3200 – 3700 cm⁻¹ in the infrared spectrum of methylated OMZ 51 B polysaccharide is indicative of complete methylation. The molar proportions of the methylation analysis products are given in table III 3.

Table III 3 The molar proportions of the products of the methylation analysis of OMZ 51 B polysaccharide

<table>
<thead>
<tr>
<th>Partially acetylated methyl alditol</th>
<th>Molar proportion</th>
<th>⁷-TMG</th>
<th>⁷-TMG standards</th>
</tr>
</thead>
<tbody>
<tr>
<td>1,5-di-Ω-acetyl-2,3,4,6-tetra-Ω-methyl-D-glucitol</td>
<td>14.5%</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>1,5,6-tri-Ω-acetyl-2,3,4-tri-Ω-methyl-D-glucitol</td>
<td>72.0%</td>
<td>2.2</td>
<td>2.2</td>
</tr>
<tr>
<td>1,3,5,6-tetra-Ω-acetyl-2,4,3i-Ω-methyl-D-glucitol</td>
<td>13.4%</td>
<td>4.2</td>
<td>4.2</td>
</tr>
</tbody>
</table>

The fragmentation patterns under electron impact and the origins of these partially acetylated methyl alditols are shown in figures II 3 – II 5 and II 7 – II 11.
The OMZ 51 B polysaccharide is essentially a polyglucan and the proportions of di-\( \alpha: \alpha - \text{tri-} \alpha - \text{tetra-} \alpha - \text{methyl-} \beta - \text{glucitol derivatives in the methylation analysis product is (within experimental error) } 1:5:1.

Thus, the average repeating unit of OMZ 51 B polysaccharide, as determined by methylation analysis, is as represented in figure III 1.

**Figure III 1** The average repeating unit of OMZ 51 B polysaccharide

\[
\begin{array}{c}
\text{O} \\
\rightarrow \\
\rightarrow \\
\rightarrow \\
\rightarrow \\
\rightarrow \\
\end{array}
\]

III B 4 The enzymic degradation of OMZ 51 B polysaccharide by

\( \text{P. lilacinum dextranase} \)

OMZ 51 B polysaccharide was hydrolysed using the \( \text{P. lilacinum} \) dextranase as described (VIB 6).

A quantitative digest showed hydrolysis to be complete after 10 h. It has been demonstrated that the extent of degradation of dextrans by the fungal dextranase decreases with increasing degrees of branching of the polysaccharides.

Comparison of the extent of degradation of OMZ 51 B polysaccharide shows it to be more extensively degraded (c. 20\%) than the more highly branched GS-5 B polysaccharide (c. 12\%).
Paper chromatography of the qualitative digest shows the absence of a 'branch' tetrasaccharide in the enzymic hydrolysate. These findings are in agreement with the results of the methylation analysis of OMZ 51 B (IIIB 3) which shows that all the secondary linkages occur at branch points in the polysaccharide structure.

IIIB 5 The determination of the minimum length of the external branch chains in OMZ 51 B polysaccharide by swine kidney glucan-hydrolase hydrolysis studies

OMZ 51 B polysaccharide was enzymically degraded and the hydrolysate was analysed as described (VIB 7). The results are shown in figure III 3.

The extent of degradation (approximately 13%) is indicative of a minimum average external chain length of 2 (correct to the nearest whole number).

Thus the average repeating unit of OMZ 51 B polysaccharide is as shown in figure III 2.

Figure III 2 The revised average repeating unit of OMZ 51 B polysaccharide
Fig. 111.3 The enzymic degradation of OMZ 51 A and OMZ 51 B polysaccharide by swine kidney glucanhydrolase.
The molecular weight distribution of OMZ 51 B polysaccharide determined by gel permeation chromatography on Sephadex G-200 (VIB 8) is shown in figure III 4. The polysaccharide was shown to be polydispersed with $M_W = 32,000$. 

IIIB 6 The molecular weight distribution of OMZ 51 B polysaccharide determined by gel permeation chromatography
Fig. 111 4 The elution diagram of CMZ 51 B polysaccharide on Sephadex G-200

\[ \bar{M}_W = 32,000 \]
IIIC The OMZ 51 A polysaccharide

IIIC 1 Determination of the monosaccharide components by acid hydrolysis

A small quantity of the polysaccharide was hydrolysed under conditions designed to completely cleave glucosidic linkages in the polysaccharide with the minimum degradation of the glucose released (VIB 9). The chromatographic properties of the product of hydrolysis are shown in table VI 29. The hydrolysate when subjected to paper chromatography in solvent (d) gave only a single spot migrating as glucose. The intensities of the glucose spots obtained from both fractions of OMZ 51 polysaccharide appeared similar by visual inspection.

A sample of OMZ 51 B polysaccharide was hydrolysed under mild conditions designed to cleave fructosidic linkages. A spot migrating as glucose and a trace of a spot migrating as fructose were observed when the hydrolysate was subjected to paper chromatography in solvent (d) (table VI 30).

IIIC 2 The composition of OMZ 51 A

Quantitative estimations of the carbohydrate and non-carbohydrate components of OMZ 51 A polysaccharide were conducted (VIB 11). The results are shown in table III 4.
Table III 4  The composition of OMZ 51 A polysaccharide

<table>
<thead>
<tr>
<th>Carbohydrate content</th>
<th>Glucose content</th>
<th>Fructose content</th>
<th>Protein content</th>
<th>Ash content</th>
<th>Specific rotation ([\alpha]_{D}^{25} + 1.56^0, \text{M NaOH})</th>
</tr>
</thead>
<tbody>
<tr>
<td>%</td>
<td>%</td>
<td>%</td>
<td>%</td>
<td>%</td>
<td></td>
</tr>
<tr>
<td>97.3</td>
<td>100.5</td>
<td>0</td>
<td>2.2</td>
<td>0.5</td>
<td>+ 172°</td>
</tr>
</tbody>
</table>

III.3 Types and percentages of the variously linked D-glucose residues in OMZ 51 A polysaccharide as determined by methylation analysis

The methylation analysis of OMZ 51 A polysaccharide was performed as described (VIB 12). The relevant g.l.c. trace and mass spectra are shown in figures VI 50 - VI 54 and the infrared spectrum of the methylated polysaccharide is shown in figure VI 55.

The absence of an absorption band in the region 3200 - 3700 cm\(^{-1}\) in the infrared spectrum of methylated OMZ 51 A polysaccharide is indicative of complete methylation. The molar proportions of the methylation analysis products are given in table III 5 and the fragmentation patterns under electron impact and the origins of these partially acetylated methyl alditols are shown in figures II 3 - II 5, II 7 - II 11, II 27 and II 28.

Table III 5  The molar proportions of the products of the methylation analysis of OMZ 51 A polysaccharide

<table>
<thead>
<tr>
<th>Products of methylation analysis</th>
<th>Molar proportions</th>
<th>(T_{\text{TMG}})</th>
<th>(T_{\text{TMG}}) standards</th>
</tr>
</thead>
<tbody>
<tr>
<td>1,5-di-O-acetyl-2,3,4,6-tetra-O-methyl-D-glucitol</td>
<td>21.1</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>1,3,5-tri-O-acetyl-2,4,6-tri-O-methyl-D-glucitol</td>
<td>3.7</td>
<td>1.73</td>
<td>1.8</td>
</tr>
<tr>
<td>1,5,6-tri-O-acetyl-2,3,4-tri-O-methyl-D-glucitol</td>
<td>55.4</td>
<td>2.2</td>
<td>2.2</td>
</tr>
<tr>
<td>1,3,5,6-tetra-O-acetyl-2,4-di-O-methyl-D-glucitol</td>
<td>19.5</td>
<td>4.2</td>
<td>4.2</td>
</tr>
</tbody>
</table>
The results show that OMZ 51 A polysaccharide is essentially a polyglucan, possibly with a trace of fructose present. The proportions of di-\(\beta\): tri-\(\beta\): tetra-\(\beta\)-methyl-D-glucitol derivatives in the methylation analysis product is approximately 1:3:1.

Thus neglecting, for the moment, the presence of the 3.7% of \(\alpha-(1\rightarrow 3)\) linkages that occur at non-branch points in the polysaccharide, the average repeating unit of OMZ 51 A polysaccharide is as shown in figure III 5.

Figure III 5  The average repeating unit of OMZ 51 A polysaccharide

\[ \begin{array}{c}
\bigcirc \\
\bigcirc \rightarrow \bigcirc \rightarrow \bigcirc \\
\end{array} \]

\[ n \]

IIIC 4  The enzymic degradation of OMZ 51 A polysaccharide by

\underline{P. lilacinum dextranase}

OMZ 51 A polysaccharide was hydrolysed using the \underline{P. lilacinum} dextranase as described (VIB 13).

A quantitative digest showed hydrolysis to be complete after 5 h and the polysaccharide is 18% degraded by the fungal enzyme (figure VI 56).
Paper chromatography of the qualitative digest shows the presence of trace quantities of a 'branch'-tetrasaccharide indicating that some of the 1,3-linked anhydroglucose units may occur as isolated units within a dextran type molecule.

IIIC.5 The determination of the minimum length of the external branch chains in OMZ 51A polysaccharide by swine kidney glucan-hydrolase hydrolysis studies

OMZ 51A polysaccharide was enzymically degraded and the hydrolysate was analysed as described (VIB 14).

The results are shown in figure III 2. The extent of degradation of the OMZ 51A polysaccharide is c. 12.5%, and thus the minimum average external chain length is 1.6. The revised average repeating unit, neglecting glucose units linked through positions 1 and 3 only, is illustrated in figure III 6.

Figure III 6 The revised average repeating unit of OMZ 51A polysaccharide
In a series of initial studies, Sidebotham et al. investigated the polysaccharide elaborated by *S. mutans* OMZ 51.

The OMZ 51 B polysaccharide was shown to contain 83% of primary linkages by n.m.r. and 76% of primary and 24% of secondary linkages by periodate oxidation studies.

The OMZ 51 A polysaccharide was similarly shown to contain 79% of primary linkages by n.m.r. and 77% of primary linkages and 23% of various secondary linkages by periodate oxidation studies.

Both polysaccharides were shown to contain a small percentage (less than 3%) of fructose.

In the present study, it was demonstrated, by methylation analysis, that 86% of the anhydroglucose units in OMZ 51 B polysaccharide were (1→6) linked and that the remaining 14% were linked through positions 1, 3 and 6. The products of the methylation analysis were characterised by mass spectrometry.

The polysaccharide was readily degraded by the endodextranase prepared from *P. lilacinum*. The minimum average external chain length of OMZ 51 B polysaccharide was 2, indicating that the sequences of α-(1→6)-linked D-glucopyranosyl units situated between the non-reducing chain ends and the branch point nearest to those non-reducing chain ends were relatively short. In the GS-5 B polysaccharide, such a structure was associated with a molecule whose overall form tended toward that of the Staudinger model for amylopectin (LIII).

The polysaccharide fraction was shown to contain 6% of fructose but the exact nature of this portion of the polysaccharide was not investigated.
Polysaccharide OMZ 51 B was shown by gel permeation chromatography, to be polydispersed ($\bar{M}_w = 32,000$).

OMZ 51 A polysaccharide contained glucose as the major monosaccharide component.

It contained approximately 20% of $\alpha-(1\rightarrow3)$-linked $D$-glucopyranose units which occurred at branch points in the polysaccharide. A further, approximately 20% of the anhydroglucose units were located at terminal, non-reducing positions.

Also present in the polysaccharide OMZ 51 A was 3.7% of $\alpha-(1\rightarrow3)$-linked $D$-glucopyranose units which did not occur at branch points.

Degradation of OMZ 51A polysaccharide by the endodextranase of *P. lilacinum* produced a trace of ‘branch’ tetrasaccharide. Such a tetrasaccharide could only have arisen from isolated $\alpha-(1\rightarrow3)$-linked $D$-glucopyranose units occurring at points other than branch points in a dextran-type structure.

Swine kidney glucanhydrolase hydrolysis of OMZ 51 A polysaccharide showed that the minimum average external chain length of OMZ 51 A polysaccharide was 1.6. This was lower than that found for the soluble fraction.

However, if the isolated $\alpha-(1\rightarrow3)$-linkages detected in OMZ 51 A polysaccharide were located at non-reducing terminal positions, they might effectively render such chains stable to hydrolysis by the swine kidney glucanhydrolase.
CHAPTER IV  THE ENZYMIC HYDROLYSIS OF SEVERAL BACTERIAL POLY-
SACCHARIDES BY THE GLUCANHYDROLASE ISOLATED FROM SWINE
KIDNEY

Exodextranases i.e. enzymes that degrade dextrans in a
stepwise fashion from the chain ends have been obtained from species
of soil bacteria, Bacillus, Bacteroides, Lactobacillus, Streptococcus,
and from animal tissue, but little structural work involving such enzymes has been reported.

Rosenfeld et al. isolated from animal tissue, an
enzyme reported to cleave the $\alpha-(1\rightarrow 6)$ linkages in dextrans, in a
stepwise fashion, up to the branch points, producing glucose and
residual dextran only. There is, however, some evidence that linear
$\alpha-(1\rightarrow 3)$ linkages are also cleaved by the enzyme.

Reductions in molecular weight of 18-35% have been reported for
clinical dextrans treated with this enzyme preparation and
between 20-25% for native dextrans.

The exodextranase [\(\alpha-(1\rightarrow 6) \) glucanhydrolase] isolated from a
strain of Streptococcus mitis produced a reduction
in molecular weight of 30% in an acid-degraded L. mesenteroides
NRRL B-512 dextran and conversions to glucose of 0-34% for various
Streptococcus and Leuconostoc dextrans are reported.

Initial attempts to isolate an exodextranase from a species of
Bacillus as described by Zevenhuisen proved unsuccessful. Although
the bacterium was successfully cultured on a dextran-containing medium
attempts to isolate the enzyme without breaking the organelles of the
bacterium failed.
Fig. IV 2  The swine kidney glucanhydrolase digest of various bacterial polysaccharides

Legend

- S. mutans OMZ 52 A polysaccharide
- L. mesenteroides NRRL B-1299 S dextran
- L. mesenteroides NRRL B-1299 L dextran
- L. mesenteroides NRRL B-1415 dextran
- L. mesenteroides NRRL B-1416 dextran
- L. mesenteroides NRRL B-1405 dextran

% conversion to glucose vs. time (hours)
Fig. IV 3 The swine kidney glucanhydrolase digest of various carbohydrates

- ○ ○ S. mutans Ingbritt A
- ○ S. mutans Ingbritt B
- □ □ sucrose
- △ △ potato amylase
- △ △ S. salivarius strain S1 "levan"

% conversion to glucose

- 50

100 200 300 400

time (hours)
An exodextranase was however isolated from swine kidney and purified by ammonium sulphate precipitation (VIA 18 (b)).

Using such an enzyme system, it is possible that information concerning the fine structure of dextrans could be obtained, by methods similar to those employed for the elucidation of the fine structure of amylopectin and glycogen-type polysaccharides.\(^{449-454}\)

The isolation and some properties of the glucanhydrolase obtained from swine kidney were discussed in section IIB 11.

A number of swine kidney glucanhydrolase digests containing various bacterial dextrans and other carbohydrates as substrates were prepared and incubated at 37°C for 500 hours.

The results are illustrated in figures IV I-IV 3.

The degrees of degradation (percentages of conversion to glucose) are shown in table IV 1 along with the percentages of secondary linkages in the polysaccharides studied.

Thin layer chromatograms of the 500 hour swine kidney glucan-hydrolase hydrolysates of several carbohydrates are shown in figure IV 4.

The long incubation time necessary to produce the limit dextran could be due to several possible factors.

(a) The low initial activity of the enzyme.
(b) Inhibition of the enzyme by the glucose produced.
(c) Deactivation of the enzyme.
(d) The increasing difficulty of contact between available anhydroglucose units in terminal non-reducing positions and the enzyme molecule as the hydrolysis proceeds and the number of available anhydroglucose units in terminal non-reducing positions falls.
Purification of the enzyme would help to increase the enzyme activity and so partially overcome factor (a). Factors (b) and (c) have already been discussed in section IIB 11 and have been shown to be insignificant. Factor (d) is a property of the particular enzyme-substrate system and is likely to be the major factor influencing the low reaction rate of the system. Similar incubation periods are reported by Rosenfeld and Walker for similar systems.

Table IV 1 The percentages of conversion to glucose of various carbohydrates on incubation with the glucanhydrolase isolated from swine kidney

<table>
<thead>
<tr>
<th>Carbohydrate</th>
<th>% of secondary linkages occurring at branch points</th>
<th>% of secondary linkages occurring at non-branch points</th>
<th>Percentage conversion to glucose by swine kidney glucan hydrolase</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. mutans GS-5 A polysaccharide</td>
<td>20</td>
<td>1-4</td>
<td>8.0</td>
</tr>
<tr>
<td>S. mutans GS-5 B polysaccharide</td>
<td>17</td>
<td>-</td>
<td>15.5</td>
</tr>
<tr>
<td>S. mutans OMZ 51A polysaccharide</td>
<td>20</td>
<td>4</td>
<td>12.5</td>
</tr>
<tr>
<td>S. mutans OMZ 51 B polysaccharide</td>
<td>14</td>
<td>-</td>
<td>13.0</td>
</tr>
<tr>
<td>S. mutans Ingbritt A polysaccharide</td>
<td>-</td>
<td>(33)</td>
<td>1.0</td>
</tr>
<tr>
<td>S. mutans Ingbritt B polysaccharide</td>
<td>-</td>
<td>-</td>
<td>2.0</td>
</tr>
<tr>
<td>S. mutans OMZ 52A polysaccharide</td>
<td>-</td>
<td>-</td>
<td>13.0</td>
</tr>
<tr>
<td>L. mesenteroides NRRL B-512 dextran</td>
<td>52, 109</td>
<td>-</td>
<td>8.5</td>
</tr>
<tr>
<td>acid-degraded L. mesenteroides NRRL B-512 dextran (Dextran T40)</td>
<td>(5)</td>
<td>-</td>
<td>8.5</td>
</tr>
<tr>
<td>L. mesenteroides NRRL B-1375 (Birmingham dextran)</td>
<td>1674</td>
<td>-</td>
<td>9.0</td>
</tr>
<tr>
<td>L. mesenteroides NRRL B-1415 dextran</td>
<td>1476</td>
<td>-</td>
<td>10.5</td>
</tr>
<tr>
<td>L. mesenteroides NRRL B-1416 dextran</td>
<td>1776</td>
<td>-</td>
<td>11.5</td>
</tr>
<tr>
<td>L. mesenteroides NRRL B-1405 dextran</td>
<td>-</td>
<td>-</td>
<td>5.5</td>
</tr>
<tr>
<td>L. mesenteroides NRRL B-1299S dextran</td>
<td>3478</td>
<td>78</td>
<td>4.0</td>
</tr>
<tr>
<td>L. mesenteroides NRRL B-1299L dextran</td>
<td>3278</td>
<td>1578</td>
<td>3.5</td>
</tr>
<tr>
<td>S. salivarius strain S1 'levan'</td>
<td>-</td>
<td>-</td>
<td>&gt;30.0</td>
</tr>
<tr>
<td>potato amylose</td>
<td>-</td>
<td>-</td>
<td>&gt;55.0</td>
</tr>
<tr>
<td>sucrose</td>
<td>-</td>
<td>-</td>
<td>1.0</td>
</tr>
</tbody>
</table>

Figures in brackets ( ) are percentages of secondary linkages - type not defined.
**Fig. 1V 4 Thin layer chromatograms of the 500 hour swine kidney glucanhydrolysates of several carbohydrates**

<table>
<thead>
<tr>
<th>Glucose standard</th>
<th>S. mutans GS-5 A polysaccharide</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>S. mutans GS-5 B polysaccharide</td>
</tr>
<tr>
<td></td>
<td>S. salivarius St levan polysaccharide</td>
</tr>
<tr>
<td></td>
<td>sucrose polysaccharide</td>
</tr>
<tr>
<td></td>
<td>amylose polysaccharide</td>
</tr>
<tr>
<td></td>
<td>glucose standard polysaccharide</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Glucose standard</th>
<th>L. mesenteroides NRRL B-1415 polysaccharide</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>L. mesenteroides NRRL B-1416 polysaccharide</td>
</tr>
<tr>
<td></td>
<td>L. mesenteroides NRRL B-1405 polysaccharide</td>
</tr>
<tr>
<td></td>
<td>acid-degraded L. mesenteroides NRRL B-512 polysaccharide</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Glucose standard</th>
<th>acid-degraded L. mesenteroides NRRL B-512 polysaccharide</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. mutans OMZ 51 A polysaccharide</td>
<td>S. mutans OMZ 51 B polysaccharide</td>
</tr>
<tr>
<td>S. mutans OMZ 52 A polysaccharide</td>
<td>glucose standard</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Glucose standard</th>
<th>S. mutans Ingbritt A polysaccharide</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. mutans Ingbritt B polysaccharide</td>
<td>L. mesenteroides NRRL B-512 polysaccharide</td>
</tr>
<tr>
<td>glucose standard</td>
<td></td>
</tr>
</tbody>
</table>
The percentage conversion to glucose from each polysaccharide investigated was calculated from the amount of starting material (approximately 20 mg) in each digest (corrected for carbohydrate content) and the amount of reducing sugar (determined as glucose) released into the digest.

Examination of figure IV III shows that the swine kidney glucanhydrolase does not invert sucrose significantly, since no increase in reducing power is noted after 500 hours incubation at 37°C.

However, both _S. salivarius_ 'levan' and potato amylose are extensively degraded by the enzyme preparation. This indicates that the enzyme preparation is not specific and probably comprises a number of enzyme systems.

An external chain of a dextran can be defined as the series of \( \text{D-glucopyranosyl} \) units situated between a non-reducing chain end and a branch point.

The average external chain length of a polysaccharide can be obtained from the following formula:

\[
E = \frac{H}{B} + X
\]

where
- \( E \) is the average external chain length
- \( H \) is the percentage of enzymic hydrolysis
- \( B \) is the percentage of branching
- \( X \) is the number of units in the external chain not removed by the enzyme.

Assuming that the branches are of the type shown in structure (LV)

- \( \text{structure (LV)} \)
then at least one D-glucopyranosyl unit will not be removed by the swine kidney glucanhydrolase.

Thus, in the above equation $X > 1$ and the minimum average external chain length, $E$, is given by:

$$E = \frac{H}{E} + 1$$

The lengths of the branch chains in the polysaccharides elaborated by *S. mutans* GS-5 and *S. mutans* OMZ 51 have been discussed in sections II B 11, II C 5, II B 5 and II C 5.

The *S. mutans* Ingbritt polysaccharides (fractions A and B) are very slightly degraded by the swine kidney glucanhydrolase. Such degrees of hydrolysis are characteristic of polysaccharides in which the external chains are very short or are terminated by non-hydrolysable functions.

Sidebotham et al. showed, by periodate oxidation studies, that 33% of the Ingbritt A polysaccharide consisted of secondary-linked D-glucopyranose units. Baird et al. showed, by methylation analysis, that one strain of *S. mutans*, described as *S. mutans* strain Ingbritt A, produced a polysaccharide in which 63% of the D-glucopyranosyl units were linked through secondary positions, 51% occurring at non-branch points.

Polysaccharide structures containing such low proportions of D-glucopyranose units linked only through positions 1 and 6, would not be expected to be extensively degraded by swine kidney glucanhydrolase. The soluble and the lesser soluble polysaccharides elaborated by *L. mesenteroides* NRRL B-1299, B-1299S and B-1299L dextrans respectively, have been studied in detail by Bourne et al.
The soluble fraction was shown to contain 34% of secondary linkages occurring at branch points and 7% occurring at non-branch points, and the average repeating unit, determined from analysis of the oligosaccharides obtained from the \textit{P. lilacinum} digest of the partially-acid-degraded B-12998 dextran, was as shown in figure IV 5.

\textbf{Figure IV 5} The average repeating unit of B-12998 dextran—possible structure

\[ \text{(LVI)} \]

In the light of the present study, several points are of particular interest in such a structure. Any branch terminated by a secondary-linked D-glucopyranosyl unit would not be degraded by the swine kidney glucanhydrolase. Furthermore, such a structure contains branches that can be represented by partial structure (LVI).
i.e. where the polysaccharide chain is continued via a secondary linkage, and the branch consists of (at least) one D-glucopyranosyl unit linked \( \alpha-(1\rightarrow6) \) to the main polysaccharide chain. In such a structure the branch might be hydrolysed down to the branch point i.e. \( X > 0 \), and the minimum average external chain length, \( E \), is given by:

\[
E = \frac{N}{D}
\]

Thus, the minimum average external chain length of \( B-1299S \) dextran is 0.9, which is in good agreement with the findings of Jeanes \(^{110}\), who demonstrated, by statistical analysis of the products of acid hydrolysis of this polysaccharide, that most of the branches were only one unit long.

When the lesser-soluble fraction, \( B-1299 L \) dextran, was treated with swine kidney glucan hydrolase, a similar percentage conversion to glucose (3.5\%) was reported. It is assumed therefore that the two polysaccharide fractions are broadly similar in structure.

Lindberg et al. \(^{142}\) showed, by a method involving the modification of the primary carbinol groups of dextrans and the subsequent degradation of the modified polysaccharide, that 40\% of the branches in \( L. \) mesenteroides NRRL B-512 dextran consisted of a single \( D \)-glucopyranosyl unit and that a further 45\% consisted of two \( D \)-glucopyranosyl units.

Smith degradation studies \(^{96}\) and methylation analysis \(^{102}\) showed that the polysaccharide contained 5\% of secondary linkages and all occurred at branch points.

When the \( L. \) mesenteroides NRRL B-512 dextran was treated with
the swine kidney glucanhydrolase, 8.5% of the polysaccharide was converted to glucose. Thus the minimum average external chain length of this polysaccharide is 2.7. Thus the minimum average length of the remaining 15% of the branches in the \textit{L. mesenteroides} NRRL B-512 dextran is approximately 9.

This contrasts with a minimum average chain length of 31 for the remaining 15% of the branches in \textit{L. mesenteroides} NRRL B-512 dextran, obtained by using the percentage of hydrolysis reported by Walker et al\textsuperscript{154}, using \textit{S. mitis} $\alpha$-(1→6) glucanhydrolase as the exodextranase. Such a structure, i.e. one in which 15% of the chains in \textit{L. mesenteroides} NRRL B-512 dextran comprise on average, at least 31 contiguous, non-branching, $\alpha$-(1→6)-linked $\beta$-glucopyranosyl units, would appear unlikely considering the mode of action of the synthesising enzyme system. It would thus seem possible that the $\alpha$-(1→6) glucanhydrolase prepared by Walker et al\textsuperscript{125-127} is able to by-pass some of the branch points in dextrans.

Swine kidney glucanhydrolase degradation of Dextran T40 (acid-degraded \textit{L. mesenteroides} NRRL B-512 dextran), reported to have a degree of branching of 5%, produced a percentage conversion to glucose of 8.5%, similar to that reported for the native polysaccharide.

Thus, the acid degradation, used to prepare Dextran T40, would appear to cleave linkages in the main chain of the polysaccharide rather than debranch it or hydrolyse primary linkages located in branch chains.
The *Leuconostoc* dextrans produced by *L. mesenteroides* NRRL B-1375 (Birmingham strain), *L. mesenteroides* NRRL B-1415, and *L. mesenteroides* NRRL B-1416 have been shown to contain 16%, 14% and 17% respectively, of secondary linkages all of which occur at branch points.

Swine kidney glucanhydrolase hydrolysis of these dextrans produced conversions to glucose of 9%, 10.5% and 11.5% respectively.

Thus, the minimum average external chain lengths are approximately 1.5, 1.75 and 1.7 respectively.

These results are in good agreement with those of Abbott et al. who showed, by periodate oxidation studies, that the majority of the branches in *L. mesenteroides* NRRL B-1415 and NRRL B-1416 dextrans consisted of a single D-glucopyranosyl unit.

In this present series of enzymic hydrolysis experiments, it is acknowledged that the enzyme preparation used probably represents a mixture of several enzyme systems.

This series of experiments however, which should be regarded as an initial study and the basis for further investigations, serves to demonstrate the important information that can be obtained from the exodextranase hydrolysis of dextrans, concerning the 'fine structure' of these polysaccharides.
CHAPTER V  

GENERAL METHODS

V A Chromatography

V A 1 Paper chromatography

Paper chromatography was carried out by the descending technique using Whatman No.1 paper for general qualitative work and No.3 and No.17 papers for preparative work (medium and large scale, respectively).

The maximum loadings for the two paper types were 100 mg per standard sheet for No.3 papers and 1000 mg per standard sheet for No.17 papers.

No.17 papers were fitted with a wick of No.3 paper and all preparative papers were washed before use with distilled water (500 ml).

Two methods for the location of compounds on preparative papers were employed.

(i) After developing the papers in the chosen solvent system, five one centimeter strips were cut from the air-dried paper and these were sprayed with, or dipped in, the appropriate staining reagent. The position of the bands in these strips was then used to locate the material in the body of the paper.

(ii) After developing the paper in the chosen solvent system and while the paper was still damp, it was laid on a clean sheet of glass and a sheet of No.1 paper laid upon it. An imprint was taken by running a blunt-edged implement up and down the paper several times. The No. 1 paper was then dipped or sprayed and the distribution of compounds in the original chromatogram was obtained.

The strips containing the separated material were cut from the chromatogram, stapled to paper wicks, and the compound was eluted from the paper with distilled water.
The solvent systems used for paper chromatography were as follows:

(a) n-butanol:pyridine:water (6:4:3 by volume)\textsuperscript{458}.
(b) ethyl acetate:pyridine:water (upper layer 2:1:2 by volume)\textsuperscript{459}.
(c) n-butanol:ethanol:water (40:11:19 by volume)\textsuperscript{460}.
(d) ethyl acetate:acetic acid:formic acid:water (18:3:1:4 by volume)\textsuperscript{461}.

V A 2 Electrophoresis

The electrolytes used for electrophoresis were as follows:

(a) sodium borate electrolyte, pH 10.0\textsuperscript{428} \( (0.05 \text{ M}) \)

The electropherograms were run at 2 Kv for 1.5 h on Whatman No.3 paper on a Shandon L24 high voltage apparatus. The standard migrating marker used was \( \text{D-} \)glucose and the non-migrating marker employed was 2,3,4,6-tetra-\( \text{D-} \)methyl-\( \text{D-} \)glucose.

(b) sodium molybdate electrolyte, pH 5.0\textsuperscript{429} \( (0.1 \text{ M}) \)

The electropherograms were run at 1.5 Kv for 2.5 h on Whatman No.3 paper on a Shandon L24 high voltage apparatus. The standard migrating marker used was \( \text{D-} \)glucitol and the standard non-moving marker was glycerol.

V A 3 Thin layer chromatography

Thin layer chromatography was performed on Camlab Polygram Sil G pre-coated thin layer chromatography plates (coated with silica gel without gipsum). The solvent systems employed were:

(a) benzene:methanol (9:1 by volume).
(b) n-butanol:acetic acid (glacial):water (upper layer 4:1:5 by volume)\textsuperscript{462}. 
The developed plates were stained by spraying with conc. sulphuric acid:ethanol:water (5:47:47 by volume).

They were then heated in an oven at 120°C for five minutes to allow the colour to develop.

**V A 4 Staining reagents**

The following staining reagents were employed for the location and identification of compounds on paper chromatograms and electropherograms.

(a) **Silver nitrate dip**

The developed paper was passed sequentially through solutions of saturated aqueous silver nitrate (2.5 ml) in water (10 ml) with acetone (500 ml), sodium hydroxide (20 g) in water (40 ml) with ethanol (960 ml), and sodium thiosulphate (10 g) in water (100 ml). Air drying of the papers was allowed between dips.

(b) **p-Anisidine hydrochloride spray reagent**

The developed paper was sprayed with a solution of p-anisidine hydrochloride (0.5 g) in ethanol (10 ml) with n-butanol (40 ml).

(c) **Aniline, diphenylamine, phosphoric acid spray reagent**

The developed paper was sprayed with a solution containing diphenylamine (4 g), aniline (4 ml), and 85% phosphoric acid (20 ml) in acetone (200 ml).

(d) **Aniline oxalate spray reagent**

The developed paper was sprayed with a solution containing aniline oxalate (25 g) in 5% aqueous ethanol (1 l).
(e) Urea hydrochloride spray reagent

The developed paper was sprayed with a solution containing urea (10 g) in ethanol (200 ml) to which conc. hydrochloric acid (8 ml) in water (32 ml) had been added.

To develop the colour, papers treated with reagents (b), (c), (d) and (e) were heated at 105°C for 5-15 mins.

V A 5 Gas-liquid chromatography (g.l.c.)

(a) Packing materials

Two packing materials were employed.

(i) 3% (w:w) ECNSS-M on Chromosorb W (100-120 mesh) (silane treated).

(ii) 3% (w:w) OV 225 on Gas Chrom Q (100-120 mesh).

To prepare the packing materials, the stationary phase (3 g) was dissolved in chloroform A.R. (25 ml). The inert support (97 g) was placed in a large evaporating dish and an aliquot of the solution was spotted in. The whole was then stirred thoroughly and allowed to dry. The process was repeated until all the stationary phase had been applied.

(b) Instrumentation

G.l.c. was carried out using:

(i) The Pye 104 double column gas chromatograph with dry nitrogen as carrier gas, a flame ionisation detector (f.i.d.) and glass columns (3M x 2 mm).

(ii) The Perkin Elmer F11 gas chromatograph with dry helium as carrier gas, a f.i.d. and glass columns (2M x 1 mm). The gas
chromatograph was coupled via an all glass inlet system and a Watson Bieman separator to a Hitachi RMS 4 mass spectrometer (see Chapter VII).

V A 6 Gel permeation chromatography

(a) Sepharose columns

Sepharose 6B and 2B were obtained from Pharmacia (G.B.) Ltd. The gels were supplied in a pre-swollen state and preserved with 0.2% sodium azide. The columns used were Pharmacia K15/90 and K16/100. The space beneath the support screen of the column was filled by injecting eluant (1% sodium chloride) back along the exit tubing until all air bubbles were expelled. Since the gel, as supplied, was too viscous to allow the escape of trapped air bubbles, it was diluted 1:1 (v:v) with the eluant. The slurry was then deaerated by pumping in a closed system.

Two methods were employed for filling the columns.

(i) The zone packing method

The slurry was carefully poured down the inside of the column allowing the escape of trapped pockets of air until the column was full. The top of the column was screwed into place and the air pocket above the slurry vented, (failure to do this results in turbulence and uneven packing as the eluant drips into the column packing) and the eluant was allowed to flow at the working pressure of the column. After a period of time the sepharose had packed down to approximately half the column volume. The column was opened and most of the excess eluant removed by pipette. The surface of the packing material to a depth of about 10 cm was then vigorously
roused and the air was vented and the eluant allowed to flow as before. The process was repeated until the column was filled to within 5 cm of the top of the column.

(ii) The extension tube method

The top of the column was fitted with a funnel and a tight fitting bung. Slurry (320 ml) was carefully poured into the column, allowing the dispersion of air pockets, so filling the column and part of the funnel. This was then allowed to pack down until the level of the slurry was below the top of the column. The air pocket above the bed was vented and eluant allowed to flow, at the working pressure, until the bed was stable.

(b) Sephadex columns

Sephadex G-100 and G-200 were obtained from Pharmacia (G.B.) Ltd. as powders. They were swollen by mixing (15 g and 10 g, respectively) with excess (600 ml) eluant (1% sodium chloride) and heating on a steam bath without stirring for 12 h. The column was then filled as for Sepharose. The working pressure of the columns were 30 cm of water for G-100 and 13 cm of water for G-200. This gave flow rates of 0.5 and 0.1 ml per min, respectively.

(c) Mixed bed Sephadex G-200, Sephadex G-10 columns

Sephadex G-200 and Sephadex G-10 were obtained from Pharmacia (G.B.) Ltd. as powders. The two were mixed thoroughly (5 g of each) and excess (300 ml) of eluant (1% sodium chloride) was added and the suspension was heated on a steam bath without stirring for 12 h. The column, a Pharmacia K9/15 was then filled as for Sepharose and a working pressure of 10 cm of eluant was applied. This gave a flow rate of 0.1 ml/min.
Definitions of terms used in chromatography

(a) Terms used in paper and thin layer chromatography

\[ R_F = \frac{\text{distance from base line moved by the component}}{\text{distance from base line moved by the solvent front}}. \]

\[ R_X = \frac{\text{distance from base line moved by the component}}{\text{distance from base line moved by the standard component X}}. \]

\[ R_M = \log_{10} \left( \frac{1 - R_F}{R_F} \right) \]

(b) Terms used in paper electrophoresis

\[ M_X = \frac{\text{distance from non-moving marker moved by a component}}{\text{distance from the non-moving marker moved by a standard compound X}}. \]

In sodium borate electrolyte the standard compound was \( \text{D-glucose} \) and the non-moving marker \( 2,3,4,6\)-tetra-\( \text{O-methyl-D-glucose} \).

In sodium molybdate electrolyte the standard was \( \text{D-glucitol} \) and the non-moving marker was glycerol.

(c) Terms used in gas liquid chromatography (g.l.c.)

\[ T_X = \frac{\text{time taken for the component to pass through the column}}{\text{time taken for the standard compound X to pass through the column}}. \]

For g.l.c. of methylated alditol acetates the standard compound X was \( 1,5\)-di-\( \text{O-acetyl-2,3,4,6-tetra-O-methyl-D-glucitol} \).

(d) Terms used in gel permeation chromatography

\( V_e \), the elution volume of a substance is that volume of eluant that must be passed through the column in order to elute
the substance in question, and it is measured as the volume between the point of sample application and the maximum of the peak in the elution diagram.

$V_o$, the void volume (the dead volume) is the elution volume of a substance that is completely excluded from the gel and it is identical to the volume of the interstitial liquid between the gel grains in the bed.

$V_t$, the bed volume (the total volume) is the total volume of the bed.

**V B Bacteriological techniques**

**V B 1 General bacteriological techniques**

(a) **Innoculations**

Innoculations were made with either pre-sterilized, cotton wool-plugged pipettes, the tips of which had been 'flamed' (i.e. passed through a roaring bunsen flame) before inoculation, or by using 'Steriseal' pre-sterilized, disposable syringes and needles. All inoculations were carried out in an ultraviolet-irradiated sterile cabinet produced in our own workshop.

(b) **Flasks and tubes**

Flasks and tubes were sealed with cotton wool plugs and both the plugs and the necks of the flasks were 'flamed' at every transfer.

(c) **Sterilizing**

All media and glassware was steam sterilized at 15 p.s.i. for 15-20 min.
(d) **Concentration of bacteria**

To determine the relative concentration of bacteria in a liquid culture, the optical density of a suitably diluted aliquot of the medium was read at 650 nm on the Unicam SP 500 spectrophotometer.

**V B 2 Media**

**M1 Recovery medium**

- Todd-Hewitt broth concentrate (Oxoid) 10 tablets
- Glucose A.R. 1 g
- Distilled water 100 ml

The minimum culture size employed was 10 ml.

**M2 Maintenance medium**

- Brain-heart infusion concentrate (Oxoid) 5 tablets
- Thioglycolate medium (w/o indicator or dextrose) (Bacto) 2.4 g
- Glucose A.R. 0.5 g
- Distilled water 100 ml

**M3 Polysaccharide production medium**

- Tryptone (Bacto) 30 g
- Yeast extract powder (Oxoid) 15 g
- Dipotassium hydrogen phosphate 9 g
- Sucrose A.R. 150 g
- Distilled water 3 l
<table>
<thead>
<tr>
<th>Medium</th>
<th>Formula</th>
<th>Components</th>
</tr>
</thead>
<tbody>
<tr>
<td>M1 Nutrient agar</td>
<td></td>
<td>Nutrient agar (Oxoid) 2.8 g</td>
</tr>
<tr>
<td></td>
<td></td>
<td>The medium was poured into 'universal' culture bottles, autoclaved, and sloped whilst still warm.</td>
</tr>
<tr>
<td>M5 Czapek Dox Agar</td>
<td></td>
<td>Czapek Dox liquid medium (modified) (Oxoid) 3.34 g</td>
</tr>
<tr>
<td>M6 Dextran-yeast extract medium</td>
<td></td>
<td>Yeast extract powder (Oxoid) 1.5 g</td>
</tr>
</tbody>
</table>
| M7 Dextran-salt medium | | (NH₄)₂SO₄ 30 g | CaCO₃ 25 g | K₂HPO₄ 10 g | MgCl₂ 2 g | S. bovis strain I dextran 10 g | Tap water 11
M8 Dextran-minimal salt medium

KH2PO4 1.5 g
(NH4)2HPO4 7.0 g
MgSO4·7H2O 0.5 g
CaCl2·2H2O 0.3 g
MnSO4·4H2O 40 mg
FeSO4·7H2O 2.5 mg
(NH4)6Mo7O24·4H2O 2.0 mg
Distilled water 1 l

The pH of the medium was adjusted to pH 7.0 with conc. hydrochloric acid. The medium was boiled and filtered. S. bovis dextran (10 g) was autoclaved separately and added aseptically.

V B 3 Buffers

B1 0.05M Citrate buffer pH 5.0

Two stock solutions were prepared.

(a) 0.1M citric acid (21.01 g in water [1000 ml]).

(b) 0.1M sodium citrate (29.41 g of C6H5O7Na3·2H2O in water [1000 ml]).

20.5 ml of (a) and 29.5 ml of (b) were mixed and the whole diluted to a total of 100 ml.

B2 0.05M citrate buffer pH 6.8

Two stock solutions were prepared.

(a) 0.1M citric acid (21.01 g in water [1000 ml]).

(b) 0.1M sodium citrate (29.41 g of C6H5O7Na3·2H2O in water [1000 ml]).

11.8 ml of (a) and 38.2 of (b) were mixed and the whole diluted to a total of 100 ml.
B3 Phosphate–citrate buffers pH range 3.4–7.0

Two stock solutions were prepared.

(a) 0.3M citric acid (21.01 g in water [1000 ml]).

(b) 0.6M dibasic sodium phosphate (53.65 g of Na₂HPO₄·7H₂O in water [330 ml]).

x ml of (a) and y ml of (b) were mixed and the whole diluted to a total of 100 ml.

<table>
<thead>
<tr>
<th>pH</th>
<th>x</th>
<th>y</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.4</td>
<td>35.9</td>
<td>14.1</td>
</tr>
<tr>
<td>4.0</td>
<td>30.7</td>
<td>19.3</td>
</tr>
<tr>
<td>4.6</td>
<td>26.7</td>
<td>23.3</td>
</tr>
<tr>
<td>5.2</td>
<td>23.3</td>
<td>26.7</td>
</tr>
<tr>
<td>5.8</td>
<td>19.7</td>
<td>30.3</td>
</tr>
<tr>
<td>6.4</td>
<td>15.4</td>
<td>34.6</td>
</tr>
<tr>
<td>7.0</td>
<td>6.5</td>
<td>43.6</td>
</tr>
</tbody>
</table>

B4 0.05M Phosphate buffer pH 5.9

Two stock solutions were prepared.

(a) 0.1M potassium dihydrogen phosphate (13.61 g of K₂HPO₄ in water [1000 ml]).

(b) 0.1M sodium hydroxide (4.0 g of NaOH in water [1000 ml]).

50 ml of (a) and 4.6 ml of (b) were mixed and the whole diluted to a total of 100 ml.
B5 0.5M Acetate buffer pH 5.0

Two stock solutions were prepared.

(a) 1.0M acetic acid (57.75 ml of glacial acetic acid in water [1000 ml]).

(b) 1.0M sodium acetate (82.0 g of C₂H₃O₂Na in water [1000 ml]).

14.8 ml of (a) and 35.2 ml of (b) were mixed and the whole diluted to a total of 100 ml.

B6 0.05M tris(hydroxymethyl)aminomethane ('tris') buffer

Two stock solutions were prepared.

(a) 0.1M 'tris' (24.2 g of 'tris' in water [1000 ml]).

(b) 0.2M hydrochloric acid.

50 ml of (a) and 41.4 ml of (b) were mixed and the whole diluted to a total of 100 ml.

B7 0.005M citrate buffer pH 6.0

Sodium citrate (C₆H₅O₇Na₃·2H₂O) (1.47 g) was dissolved in water (1 l) and the pH was adjusted to pH 6.0 with citric acid.

V B 4 Biological stains

S1 The Gram stain

Three solutions were prepared.

(a) Methyl violet 6B (Jensen methyl violet [0.5 g] in water [100 ml]).

(b) Lugol's iodine (crystal iodine [1 g] and potassium iodide [2 g] in water [100 ml]).

(c) Neutral red (Jensen neutral red [0.1 g] and 1% acetic acid [0.2 g] in water [100 ml]).
The bacterial culture was spread on a microscope slide and allowed to air dry. The slide was then 'fixed' by passing it through a bunsen flame.

The culture was stained for 20–30 sec with solution (a) which was then poured off. The slide was washed with sterile water. Lugol's iodine (solution (b)) was then applied and this was allowed to act for 30–60 sec before being poured off. Excess stain was removed by washing the slide in absolute ethanol until no more colour was removed. The slide was washed with sterile water and air dried.

Counterstaining was performed with neutral red (solution (c)) for 30–60 sec, followed by washing, blotting and air drying.

Gram positive bacteria appear blue on the slides and Gram negative bacteria appear red.

**S2 Alcian blue**

A stock solution was prepared as follows:

Alcian blue (1 g) was dissolved in ethanol (100 ml). The solution was stored at 4°C.

To stain bacterial polysaccharides, the stock solution (50 ml) was diluted with distilled water (50 ml). This solution deteriorates on keeping and was discarded after 24 h.

The bacterial culture was spread on a slide, allowed to air dry, and fixed. The staining solution was carefully applied, permitted to act for 60 sec and was then washed off with distilled water. The slides were air dried.

Counterstaining was achieved with carbol-fuchsin which was
washed off with water immediately after application. The cultures were mounted in Curr's Canada balsam under cover slips. Bacterial cells appear red and the bacterial polysaccharides blue.

**VC Physical Techniques**

**VC 1 Evaporations**

Evaporations were carried out under reduced pressure at temperatures between 30 and 45°C, unless otherwise stated.

**VC 2 Dialysis**

Dialysis was performed in Visking tubing against distilled water or an appropriate buffer.

**VC 3 Concentration of enzyme solutions**

Enzyme solutions were concentrated using an Amicon model 202 concentration cell with a UM 2 pad. (cut-off point \( M_w = 1000 \)).

**VC 4 Water**

The water used in all experiments was distilled or deionised unless otherwise stated.

**VC 5 Optical rotations**

Optical rotations were determined in a 1 dm polarimeter cell using a Perkin-Elmer 141 polarimeter. All measurements were made in \( M_w \) sodium hydroxide solution using the sodium D line.

**VC 6 Melting points**

Melting points were determined on a Gallenkamp micro melting point apparatus.
VC 7 Resins

All resins were Amberlite, unless otherwise stated.

VC 8 Freeze-drying

All freeze-drying was performed on a Chemlab laboratory freeze-drier.

VD Assays and Analyses

VD 1 Ash, nitrogen, moisture and methoxyl contents

These were determined by A. Bernhardt, Germany, on request unless otherwise stated.

VD 2 Carbohydrate contents

Carbohydrate contents were assayed by the phenol-sulphuric acid method.\(^477\)

4% phenol A.R. solution (1 ml) was added to water (1 ml) containing 10–100 \(\mu g\) of sugar. Concentrated sulphuric acid (5 ml) was rapidly added. The tube was shaken and allowed to cool for 30 min. A blank, containing water, phenol and acid only, was prepared simultaneously. The optical densities were measured at 487 nm on a Unicam SP 500 spectrophotometer.

Standard graphs were prepared based upon solutions of glucose, fructose, or a mixture of both.

VD 3 Reducing sugar contents

These were determined by using the Nelson copper reagents \(^478,479\). Three reagents were prepared.

(a) Anhydrous sodium carbonate (25 g), sodium potassium tartrate (25 g), sodium bicarbonate (20 g) and anhydrous sodium sulphate (200 g) were dissolved in water (800 ml). The solution was
diluted to 1 l and filtered if necessary.

(b) Cupric sulphate pentahydrate (30 g) was dissolved in water (200 ml) containing concentrated sulphuric acid (4 drops).

(c) Ammonium molybdate (25 g) was dissolved in water (450 ml) to which concentrated sulphuric acid (21 ml) had been added. Sodium arsenate heptahydrate (3 g) was dissolved separately in water (25 ml) and was added slowly to the above solution with constant stirring. The whole was diluted to 500 ml with water and placed in an incubator at 37°C overnight.

The solutions were stored at 4°C.

(d) A fourth solution was prepared by adding reagent (b) (1 ml) to reagent (a) (25 ml) with shaking, immediately before use. Reagent (d) (1 ml) was added to water (1 ml) containing carbohydrate (equivalent in reducing sugar content to 25-250 µg of glucose) in a pyrex test tube. A blank, containing water and reagent (d) only, was prepared simultaneously.

The tubes were placed in a boiling water bath for exactly 20 min and they were then cooled for 5 min in running tap water. Reagent (c) (1 ml) was then added, and the tubes were shaken until carbon dioxide was no longer evolved. The solutions were allowed to stand for 10 min before diluting to 25 ml with water. The optical densities of the solutions were measured at 520 nm on a Unicam SP 500 spectrophotometer. A standard curve based upon glucose was prepared.

V D 4 Glucose contents

(a) Free glucose in solution

The method used employed the blood sugar determination
method of Boehringer Biochemicals Ltd.

Bottle 2 (glucose oxidase-peroxidase reagent) of the Boehringer blood sugar kit TBA6 15755 was made up to 300 ml taking great care to avoid air bubbles in the solution. The solution was stored at 4°C and can be kept for 3 weeks.

The solution (5 ml) was pipetted into a tube containing water (1 ml) containing 5-50 μg of free glucose. The contents of the tubes were mixed by inversion and the tubes were placed in the dark for 0.5 – 1 h to allow the green colour to develop. The optical densities were measured at 436 nm on a Unicam SP 500 spectrophotometer.

A blank containing only water and the glucose oxidase-peroxidase reagent was produced simultaneously. A standard graph based upon glucose was prepared.

(b) Glucose in a chemically bound state within a polysaccharide

The polysaccharide (approx 50 mg [weight accurately known]) dried for 2 days in vacuo at 40°C, was hydrolysed for 8 h at 100°C with 3 ml sulphuric acid in a capped flask. Two separate samples of glucose A.R. were similarly treated as a check on degradation during hydrolysis. The solutions were then neutralised with 2M sodium hydroxide (3 ml) and made up to 100 ml, 1 ml of this solution was then diluted, to 50 ml.

The glucose was then estimated as in V D 4 (a).

(c) Qualitative determination of free glucose in solution

The presence of glucose in solutions was demonstrated using Worthington Glucostat X4 glucose reagents.
To a solution (1 ml) containing at least 5 \( \mu \text{g} \) of free glucose a spatula tip of both the Glucostat X4 reagent and the chromogen for the above (Worthington) was added. The solution was incubated at 37°C for 1 h. On acidification with \( 9 \text{M} \) sulphuric acid, a pink coloration indicated the presence of free glucose.

**V D 5 Fructose contents**

The fructose contents of polysaccharides were determined by the method of Wise et al.\(^{480}\)

The alcoholic anthrone reagent was prepared as follows.

Concentrated sulphuric acid (100 ml) was added slowly to absolute ethanol (50 ml) with cooling. When the mixture returned to room temperature anthrone (200 g) was added, with stirring. The reagent was ready to use and could be stored at 4°C for three weeks, after which time deterioration was indicated by coloration.

Four solutions were prepared.

(a) A solution of the polysaccharide under investigation, containing polysaccharide (approx. 1.5 mg [weight accurately known]) in water (2 ml).

(b) A solution of a standard dextran, containing Betacoccus dextran (Glaxo and Co. Ltd.) having the same concentration of polysaccharide in water (2 ml) as solution (a).

(c) A fructose standard solution, containing fructose A.R. (112 \( \mu \text{g} \)) in water (2 ml).

(d) Water (2 ml).

The tubes were cooled in ice water (to stop random reactions at room temperature) and the cold anthrone reagent (8 ml) was layered
on. The solutions were mixed by inversion and transferred to a water bath at 50°C ± 0.5°C for exactly 20 min. The solutions were then cooled for 1 minute in an ice bath and rapidly transferred to 1 cm curettes. The optical densities of the solutions were read on a Unicam SP 500 spectrophotometer at 620 nm.

Some colour develops during this assay due to the presence of glucose. To minimise this effect the tubes were read (a) to (d) and then (d) to (a) and the average values calculated.

The percentage of fructose was calculated from the following expression.

\[
\text{Wt of fructose} = \frac{\text{mg of fructose in soln.}(c) \times (\text{absorbance soln.}(a) - \text{absorbance soln.}(b) \times \frac{G}{100})^{18}}{\text{absorbance of solution (c)}}
\]

where \( G \) = glucose content of test sample as %.

V D 6 Protein contents

(a) The protein content of a polysaccharide

The protein content of a polysaccharide was obtained by multiplication of the nitrogen content of that polysaccharide by a factor (6.24)\(^{481}\).

(b) The protein contents of solutions

Estimation of the protein contents of solutions was carried out by the biuret test of Comall\(^{482}\) which is a modification of the methods of Robinson\(^{483}\) and Weichselbaum\(^{484}\).

The biuret reagent was prepared as follows.

Cupric sulphate heptahydrate (1.5 g) and sodium potassium tartrate tetrahydrate (6.0 g) were dissolved in water (500 ml).
10% sodium hydroxide solution (prepared from stock, carbonate-free, 65-75% sodium hydroxide solution) (300 ml) was added with constant swirling. The whole was diluted to 1 l with water and stored in a paraffin-lined bottle. The reagent keeps indefinitely but it was discarded if it showed any sign of depositing any black or reddish precipitate.

To a solution containing 1-10 mg/ml of protein (1 ml) the reagent (4.0 ml) was added with swirling. A blank containing only water and the biuret reagent was prepared simultaneously. The optical densities of the solutions were read at 540 nm on a Unicam SP 500 spectrophotometer after 30 minutes storage in the dark.

A standard curve based on clear serum protein was prepared from which a multiplication factor of 19.5 was obtained. Thus:

\[
\text{mg of protein} = \text{absorb. of solution} \times 19.5
\]

**V D 7. The degree of polymerisation of an oligosaccharide**

The degree of polymerisation of an oligosaccharide was determined by the Timell\(^{485}\) modification of the Peat\(^{486}\) method.

Two solutions were prepared.

(a) 2% KBH\(_4\) (0.5 ml) and the oligosaccharide (60-80 \(\mu\)g) in water (0.5 ml).

(b) 2% KBH\(_4\) (0.5 ml) and the oligosaccharide (the same weight as in soln. (a)) in \(H_2SO_4\) (0.5 ml).

The tubes were left overnight and then 4% phenol solution (1 ml) and concentrated sulphuric acid (5 ml) were added (V D 2). The optical densities of the solutions were read at 487 nm on a Unicam spectrophotometer.
V E General Reactions and Preparations

V E 1 Purification of solvents.

(a) Chloroform

Chloroform was distilled from anhydrous sodium sulphate and the distilled product was stored over anhydrous sodium sulphate in the dark, at 4°C.

(b) Dimethyl sulfoxide (d.m.s.o.).

D.m.s.o. was distilled under reduced pressure from calcium hydride and stored over 4Å molecular sieves.

(c) Iodomethane (methyl iodide)

Iodomethane was distilled at atmospheric pressure and stored over dry silver oxide in the dark, at 3°C.

(d) Super-dry methanol

Clean dry magnesium turnings (5 g) and resublimed iodine (0.5 g) were placed in a 1 l round-bottomed flask fitted with a condenser. Methanol (50-57 ml) was added and the mixture was warmed until the iodine disappeared. When hydrogen was evolved the source of heat was removed. If no hydrogen was evolved then further iodine (0.5 g) was added. Methanol (900 ml) was then added and the mixture was boiled for 30 minutes. The product was then distilled with the exclusion of water. The first 25 ml was discarded.

(e) Dry n-pentane

n-Pentane was dried with sodium wire.

(f) Pyridine

Pyridine was distilled from sodium hydroxide pellets and stored over sodium hydroxide pellets.

Wherever possible, solvents were purified immediately prior to use.
All strains of *Streptococcus mutans* were donated by Dr. W.H. Bowen of the Royal College of Surgeons of England. They were obtained in the lyophilised state and were recovered by incubating in medium M1 (V E 2) for 24 h at 37°C.

After recovery, the bacteria were maintained by subculturing through medium M2 (V E 2) for 24 h at 37°C. They were then stored at 4°C and remained viable for at least 1 month.

A 19 h old sample of this culture (5 ml) was inoculated, via the Pasteur pipette, by means of a disposable syringe, into the apparatus shown in fig. V 1 which had previously been autoclaved and allowed to cool naturally. The top of the flask was fitted with a polythene bag containing carbon dioxide, in order to increase the carbon dioxide concentration of the atmosphere of the flask at the initial stages of fermentation, and the whole was incubated at 37°C, for 90 h.

**Figure V 1** The apparatus used for polysaccharide production
After 90 h, the crude polysaccharide was extracted from the culture medium by the following scheme (figure V II).

**Figure V II** The extraction of the crude polysaccharides

**Sucrose Broth Containing Polysaccharide**
- Made 0.04M w.r.t. NaOH
- Stirred at room temp. for 20 h
- Centrifuged at 700 g for 15 min

**Supernatant**
- pH adjusted to pH 4.0
- EtOH concentration adjusted to 40% (v:v)
- Centrifuged at 700 g for 15 min

**Supernatant**
- EtOH concentration adjusted to 70% (v:v)
- Centrifuged at 700 g for 15 min

**Crude Fraction A**

**Supernatant**
- Spent medium (rejected)

**Crude Fraction B**

**Spent medium (rejected)**

**V E 3 The purification of crude polysaccharide fractions**

(a) Fraction A (the polysaccharide fraction precipitated by 40% ethanol (v:v)).

The crude polysaccharide fraction was dissolved, with stirring, in the minimum volume of water and the ethanol concentration was adjusted to 40% (v:v). The precipitate was allowed to form
(12 hr, 4°C) and the resulting suspension was then centrifuged at 700 g for 15 min. The supernatant was discarded and the precipitation was repeated.

Deproteination was achieved by the method of Sevag et al. The polysaccharide was dissolved, with stirring, in a minimum volume of water and the resulting solution was shaken with an equal volume of chloroform:amyl alcohol (7:2 v:v). The protein was seen to separate as a gel at the interface of the phases and it was removed with the organic layer. The process was repeated until no more gel formed and the aqueous layer was biuret-negative (V D 6 (b)).

The polysaccharide was reprecipitated with a further 40% (v:v) of ethanol.

The suspension was centrifuged at 700 g for 15 min. The supernatant was decanted off and discarded and the centrifugate was resuspended in water and freeze-dried.

(b) Fraction B (the polysaccharide fraction precipitated by 70% ethanol [v:v]).

The precipitations and deproteinations were conducted as for fraction A except that ethanol precipitations were conducted using ethanol concentrations of 70% (v:v).

V E 4 Acid hydrolysis of a polysaccharide

(a) Optimum conditions for the production of glucose

The polysaccharide (25 mg) was hydrolysed with H₂SO₄ sulphuric acid (2 ml) in a capped flask, on a steam bath at 100°C, for 8 h. The hydrolysate was cooled, neutralised with excess barium carbonate, and the insoluble barium salts were removed by centrifugation at 300 g for 10 min.
The supernatant was deionised by shaking with IR 120 (H⁺) resin, followed by filtration, and then with IRA 400 (OH⁻) resin, followed by filtration and concentration at reduced pressure.

(b) Optimum conditions for the production of fructose

The polysaccharide (50 mg) was hydrolysed with 0.1 M sulphuric acid (1 ml) in a capped flask, in a water bath at 70°C, for 1 h. The hydrolysate was cooled and neutralised with saturated barium hydroxide solution using bromothymol blue as indicator. The neutral solution was adjusted to give an ethanol concentration of 80% (v:v) (to remove unhydrolysed polysaccharide and barium salts) and centrifuged at 300 g for 10 min. The supernatant was concentrated at reduced pressure.

VE 5 The borohydride reduction of a monosaccharide, oligosaccharide or a hydrolysed, methylated polysaccharide

The monosaccharide, oligosaccharide or hydrolysed, methylated polysaccharide (5 mg) was dissolved in water (5 ml) and sodium borohydride (50 mg) was added. The reaction was allowed to evolve hydrogen gas at room temperature for 2 h. After treatment with IR 120 (H⁺) resin to destroy the borohydride, and evaporation to dryness in vacuo, boric acid was removed by six consecutive codistillations with methanol. The resulting solution was then evaporated to dryness in vacuo.

VE 6 The preparation of dimethylsulphinyl carbamion.

The carbamion was prepared by a slight modification of the method of Sandford et al.92

Into a dry, 300 ml three-necked, round-bottomed flask containing a magnetic stirring bar ('Teflon' coated) sodium hydride (55% coated
with mineral oil) (1.5 g) was weighed. The hydride was washed three times by stirring with 30 ml portions of n-pentane (V E 1 (e)) and decanting the wash. After the third wash, the flask was fitted with a thermometer and a stoppered condenser, and the residual n-pentane was removed by successive evacuations, through a course air bleed inserted into one neck of the flask, by a vacuum pump. After each evacuation the flask was regassed with dry nitrogen gas. When the hydride appeared totally dry the stopper was removed from the condenser and dry nitrogen was passed continually through the flask via the air bleed. Using a hypodermic syringe dimethylsulphoxide (15 ml) (V E 1 (b)) was transferred to the flask. The flask was placed on a heating block and stirred at 50°C until the solution became clear and green and evolution of hydrogen gas ceased (c. 40 min). The concentration of the carbanion in dimethylsulphoxide solution was determined by withdrawing an aliquot (1 ml) and titrating it against 0.1 M hydrochloric acid in aqueous solution.

V E 7 Methylation of polysaccharides by the Hakomori method

The polysaccharide was dried in vacuo at 35°C for 2 days before use. The polysaccharide (ca. 10 mg) in a 28 ml (1 oz) McCartney bottle was dissolved in dimethylsulphoxide (d.m.s.o) (3 ml). Dry nitrogen was flushed through the bottle and a 2 M solution of carbanion (V E 6) in d.m.s.o. (2 ml) was added dropwise from a syringe. The resulting gelatinous precipitate was agitated in an ultrasonic bath (40 KHz) for 1 h. The solution was then kept at room temperature for 6 h.
Methyliodide \((V E \ 1(c) (0.2 \ ml)\) was added dropwise with cooling, by tap water, and agitated in the ultrasonic bath for 20 min.

Further carbanion \((2 \ ml)\) was added and again the solution was ultrasonicated for 1 h and allowed to stand at room temperature for 6 h.

Methyliodide \((V E \ 1(c) (0.2 \ ml)\) was again added dropwise with cooling, by tap water, and agitated in the ultrasonic bath for 20 min.

Further carbanion \((2 \ ml)\) was added and again the solution was ultrasonicated for 1 h and allowed to stand at room temperature for 6 h.

The reaction was terminated by the addition of excess methyl iodide \((2 \ ml)\) with cooling, by tap water, and the solution was again agitated for 20 min.

The solution was poured into water \((50 \ ml)\) and dialysed against running tap water for 2 days and against distilled water for 2 days.

\[\text{V E 8 Hydrolysis of a methylated polysaccharide}\]

\(\text{(a) Using } 90\% \text{ formic acid}^{88}\)

The methylated polysaccharide \((2-10 \ mg)\) was placed in a 25 ml round-bottomed flask and a piece of 'Dri-cold' solid carbon dioxide was added and allowed to evaporate giving an atmosphere of carbon dioxide. 90% formic acid \((1 \ ml \ per \ 3 \ mg \ of \ polysaccharide)\) was added and the capped flask was heated at 100°C on a water bath for 10 h. The flask was then opened and water \((15 \ ml)\) was added and the open flask was heated at 100°C for 2 h. The product was evaporated to dryness and codistilled six times with methanol \((10 \ ml \ aliquots)\). The final solution was evaporated to dryness.
(b) **Using 72% sulphuric acid**

The methylated polysaccharide (5 mg) was placed in a 25 ml round-bottomed flask and cold 72% sulphuric acid (1 ml) was added and the reaction was left at room temperature for 1 h.

Water (8 ml) was then added. The flask was capped, and heated on a water bath at 100°C for 5 h. The mixture was cooled, neutralised with barium carbonate and filtered at reduced pressure.

**VE 9 Acetylation of a reduced, hydrolysed, methylated polysaccharide**

The reduced, hydrolysed, methylated polysaccharide was treated with acetic anhydride: pyridine (VE 1 (f)) (1:1 v:v) (1 ml per mg of starting material) and heated at 100°C on a steam bath, for 10 min, under anhydrous conditions. The acetylation mixture was evaporated to dryness and dissolved in chloroform (VE 1(a)).

**VE 10 The preparation of a dextranase from Penicillium lilacinum**

**(I.M.I. 79197)**

The organism was obtained as an agar slope culture from The Commonwealth Mycological Institute, Kew, Surrey. It was mixed with a little sterile water and looped onto plates of medium M5. The plates were incubated at 25°C for 5 days and then stored at 4°C. The organism remained viable for up to one month.

Stabs of the mycelia were suspended in sterile water (1 ml) and by the technique of sterile pouring this was transferred to three 500 ml flasks each containing medium M6 (100 ml). The flasks were incubated at 25°C for 4 days with orbital shaking.
The moulds were seen to grow as spherical floating colonies in the medium, and cultures were transferred by sterile pouring into six 500 ml flasks each containing medium M6 (200 ml). These flasks were incubated at 25°C for 7 days with orbital shaking.

The culture fluid was filtered through glass wool at reduced pressure and then through a Whatman No.3 paper to remove the insoluble material. The brown filtrate was centrifuged at 1000 g for 15 min. The supernatant was decanted off and made 0.005 M w.r.t. sodium citrate, and the pH was adjusted to pH 6.0 with citric acid.

The crude dextranase solution was dialysed against buffer B7 (6 x 4 l) and stored at 4°C under a layer of toluene A.R. The enzyme solution was later freeze-dried.
CHAPTER VI  

VI A  The polysaccharide elaborated by S. mutans GS-5

VI A 1  The production, extraction and purification of the polysaccharide

The polysaccharide was produced extracted and purified as described (VE 2 and VE 3). The yields are shown in table II 1.

VI A 2  Acid hydrolysis at optimum conditions for the production of glucose from GS-5 B polysaccharide

The polysaccharide (25 mg) was hydrolysed (VE 4(a)). After neutralisation half the product was subjected to paper chromatography in solvent (d) and paper electrophoresis in buffer (a).

The remaining half of the product was reduced (VE 5) and the reduced product was dissolved in water (0.5 ml) and subjected to electrophoresis in buffer (b). Staining reagent (a) was employed throughout.

The results are tabulated in table VI 1.

Table VI 1  Acid hydrolysis of the glucosidic linkages of GS-5 B polysaccharide – Paper chromatography and electrophoresis of the product and reduced product

<table>
<thead>
<tr>
<th>Product</th>
<th>Solvent or Buffer System</th>
<th>Value</th>
<th>Intensity with Staining Reagent (a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>R_g</td>
<td>solvent (d)</td>
<td>1.01</td>
<td>5+</td>
</tr>
<tr>
<td>M_g</td>
<td>buffer (a)</td>
<td>1.00</td>
<td>5+</td>
</tr>
<tr>
<td>M_g</td>
<td>reduced product buffer (b)</td>
<td>0.99</td>
<td>5+</td>
</tr>
</tbody>
</table>
VI A 3 Acid hydrolysis at optimum conditions for the production of fructose from GS-5B polysaccharide

The polysaccharide (50 mg) was hydrolysed (V E 4 (b)). After neutralisation and removal of unhydrolysed polysaccharide and barium salts, the hydrolysate was concentrated and half the product was subjected to paper chromatography in solvent (d) and paper electrophoresis in buffer (a).

The remaining half of the product was reduced (V E 5) and the reduced product was dissolved in water (0.5 ml) and subjected to electrophoresis in buffers (a) and (b). Staining reagents (a) and (e) (specific for ketohexoses) were employed.

The results are tabulated in table VI 2.

Table VI 2 Acid hydrolysis of the fructosidic linkages of GS-5B polysaccharide - Paper chromatography and electrophoresis of the product and the reduced product

<table>
<thead>
<tr>
<th>Solvent or buffer system</th>
<th>Value</th>
<th>Intensity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Staining reagent (a)</td>
</tr>
<tr>
<td><strong>[^]{P_g} product</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>solvent (d)</td>
<td>1.00</td>
<td>+</td>
</tr>
<tr>
<td>solvent (a)</td>
<td>1.27</td>
<td>4+</td>
</tr>
<tr>
<td><strong>[^]{M_g} product</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>buffer (a)</td>
<td>1.00</td>
<td>Trace</td>
</tr>
<tr>
<td>buffer (a)</td>
<td>0.91</td>
<td>3+</td>
</tr>
<tr>
<td><strong>[^]{M_S} reduced product</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>buffer (b)</td>
<td>1.00</td>
<td>2+</td>
</tr>
<tr>
<td><strong>[^]{M_g} reduced product</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>buffer (a)</td>
<td>0.83</td>
<td>2+</td>
</tr>
<tr>
<td>buffer (a)</td>
<td>0.92</td>
<td>+</td>
</tr>
</tbody>
</table>
VI A 4  Composition of GS-5 B polysaccharide

(a) Ash, nitrogen (protein) and moisture contents

These were determined by A. Bernhardt on request and are tabulated in table II 2.

(b) The total carbohydrate content

A solution of the polysaccharide (83.3 µg) in water (1 ml) was prepared.

The carbohydrate content of the polysaccharide solution was determined by the method of Dubois et al. (V D 2).

From the absorbances obtained (table VI 3) and by comparison with a standard graph (figure VI 1) based upon glucose, the carbohydrate content of the polysaccharide, expressed as a percentage in table II 2, was obtained.

Table VI 3  The carbohydrate content of GS-5B polysaccharide

<table>
<thead>
<tr>
<th>Vol. of polysaccharide soln. (ml)</th>
<th>Vol. of water (ml)</th>
<th>Absorbance</th>
<th>Wt. of carbo-hydrate (expt.) (µg)</th>
<th>Wt. of carbo-hydrate (theoretical) (µg)</th>
<th>Carbo-hydrate content %</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>0.5</td>
<td>.297</td>
<td>41</td>
<td>41.7</td>
<td>98%</td>
</tr>
<tr>
<td>1.0</td>
<td>0</td>
<td>.575</td>
<td>80</td>
<td>83.3</td>
<td>96%</td>
</tr>
<tr>
<td>0</td>
<td>1.0</td>
<td>Reagent blank</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Fig. VI.1 Standard graph for phenol sulphuric acid reagents

- Glucose
- 94% glucose, 6% fructose
- 49% glucose, 60% fructose
- Fructose

Absorbance

µg of glucose

0.5

50
(c) The fructose content

A solution containing the polysaccharide (1.7 mg) in water (2 ml) and a solution of Betacoccus dextran (Glaxo and Co. Ltd.) of equal concentration were prepared.

The fructose content of the polysaccharide solution was then determined by the alcoholic anthrone method of Wise et al. (V D 5). The results obtained are listed in table VI 4 and the fructose content, expressed as a percentage is shown in table II2.

Table VI 4 The fructose content of GS-5 B polysaccharide

<table>
<thead>
<tr>
<th>Solution</th>
<th>Absorbance (c)-(a)</th>
<th>Absorbance (a)-(c)</th>
<th>Average Absorbance</th>
</tr>
</thead>
<tbody>
<tr>
<td>(a) GS-5 B polysaccharide</td>
<td>0.287</td>
<td>0.285</td>
<td></td>
</tr>
<tr>
<td>(b) Standard dextran soln.</td>
<td>0.024</td>
<td>0.024</td>
<td></td>
</tr>
<tr>
<td>(c) Standard fructose soln.</td>
<td>0.733</td>
<td>0.734</td>
<td></td>
</tr>
</tbody>
</table>

(d) The glucose content

The GS-5 B polysaccharide (50.9 g) was subjected to acid hydrolysis and the glucose released was determined as described (V D 4 (b)). Two standards of glucose A.R. (48.7 g and 42.8 g) were similarly treated. The results obtained are shown in table VI 5. The glucose content, expressed as a percentage in table II 2, was calculated by comparison with a standard curve based upon glucose (figure VI 2).
Fig. VI.2 Standard curve for the glucose oxidase-peroxidase reagents.
Table VI 5  The glucose content of GS-5 B polysaccharide

<table>
<thead>
<tr>
<th>Carbohydrate</th>
<th>weight g</th>
<th>Absorbance</th>
</tr>
</thead>
<tbody>
<tr>
<td>GS-5 B polysaccharide</td>
<td>50.9</td>
<td>.226</td>
</tr>
<tr>
<td>Glucose standard 1</td>
<td>48.7</td>
<td>.210</td>
</tr>
<tr>
<td>Glucose standard 2</td>
<td>42.8</td>
<td>.184</td>
</tr>
</tbody>
</table>

(e) The optical rotation

The optical rotation of the polysaccharide was determined in M sodium hydroxide as described (VI C 5). The values of the observed rotation and the concentration are given in table VI 6 and the value of the specific rotation is given in table II 2.

Table VI 6  The optical rotation of the GS-5 B polysaccharide

<table>
<thead>
<tr>
<th>Concentration g/100 ml</th>
<th>Observed rotation [α]D 25 (c = 0.995 M NaOH)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.995</td>
<td>+1.680 +169°</td>
</tr>
</tbody>
</table>

VI A 5 The proton n.m.r. spectrum of GS-5 B polysaccharide

The polysaccharide (100 mg) was dissolved in the minimum volume (c. 1.0 ml) of deuterium oxide and allowed to stand for 15 min., in a clean round-bottomed flask fitted, using a minimum of vacuum grease, with a stopcock. The sample was then freeze-dried. This was repeated twice, and the sample was then dissolved in deuterium oxide (c. 1.0 ml).
The solution (c. 1.0 ml) was then filtered through a Millipore filter into a n.m.r. tube and the spectrum was obtained on a Varian A60 1L with an external, capillary held, standard of h.m.d.s. (hexamethyldisilane).

The spectrum obtained is shown in figure VI 3.

**VI A 6** The infrared spectrum of GS-5 B polysaccharide in the region $1000 - 600 \text{ cm}^{-1}$

The infrared spectrum of GS-5 B polysaccharide, in the region $1000 \text{ cm}^{-1} - 600 \text{ cm}^{-1}$ was obtained using a Perkin Elmer P.E. 325 infrared spectrophotometer. The sample, as a nujol mull, was held between potassium bromide plates. The spectrum obtained is shown in figure VI 4.

**VI A 7** The methylation of GS-5 B polysaccharide

(a) **Initial methylation of GS-5 B polysaccharide**

The polysaccharide (450 mg), dried in vacuo at 50°C for 3 days, was dissolved in dry distilled dimethylsulphoxide (50 ml) (VE 1(b)) in a three-necked, round-bottomed flask, containing a glass-coated magnetic stirring bar, fitted with a reflux condenser, a thermometer, and a coarse air bleed through which dry nitrogen gas was passed.

The flask was warmed slightly (c. 30°C) to aid the dissolution of the polysaccharide.

After cooling to room temperature, a 2 M solution of dimethylsulphinyl carbanion (VE 6) in d.m.s.o. (10.5 ml) was added. A green gel formed immediately. The reaction mixture was stirred at
Fig. VI 3 The NMR spectrum of deuterated GS-5 B polysaccharide

increasing value of $\tau$ (ppm)
Fig. VI 4 The infrared spectrum of GS-5 B polysaccharide
room temperature until homogeneous (80 min) and was then left to stand at room temperature for 8 h.

To achieve methylation, the polysaccharide alkoxide was cooled to 20°C in ice water and methyl iodide (3 ml) (V E 1 (c)) was added to the solution at such a rate that the temperature did not rise above 25°C. The reaction mixture was stirred until the solution became clear and the viscosity was markedly reduced (10 min). The solution was poured into water (100 ml) and dialysed against running tap water (2 days) and against distilled water (2 days). The product was extracted with chloroform (3 x 50 ml) and the extracts were evaporated to dryness at reduced pressure. The dry product was suspended in water and freeze-dried. This gave a product with superior handling properties but involved an appreciable handling loss. The freeze-acid product was further dried in vacuo at 50°C over phosphorus pentoxide.

The whole procedure was repeated twice without the extraction of the product. The methoxyl contents were obtained and the infrared spectra in the region 4000 cm⁻¹ - 2500 cm⁻¹ were investigated for the products of the various stages in the methylation procedure.

(b) Further methylation of GS-5 B polysaccharide

The apparatus as shown (figure VI 5) was tested, using a vacuum gauge, to ensure that it was air tight. Stopper 2 was removed and the apparatus was flushed with dry nitrogen gas for 5 min. Stopper 2 was replaced and the system was evacuated. The apparatus was 'flamed' by passing a hot bunsen flame over all the outer surfaces to remove any water adsorbed to the walls of the apparatus.
Sodium hydride (80 mg) (a 55% dispersion in mineral oil) was then washed with n-pentane (20 ml) (V E 1 (e)) and the n-pentane was decanted off. The washing process was repeated three times and with dry nitrogen gas flowing through the reaction vessel, the slurry of sodium hydride in n-pentane was added via a funnel inserted in the place of stopper 1. Stopper 1 was replaced, the suspension was stirred with a magnetic stirring bar, and the system was evacuated, thus pumping away the excess n-pentane. The reaction vessel was then alternately and repeatedly flushed with dry nitrogen and evacuated (6 – 10 times). Finally dry nitrogen gas was allowed to flow continually and stopper 2 was replaced by a calcium chloride drying tube.

A clean completely dry flask was flushed with dry nitrogen. The partially methylated, freeze-dried polysaccharide (200 mg) (methoxyl content = 38.4% [corrected]) was dried in vacuo at 50°C for 5 days and was then placed in the flask and dissolved in dimethyl-sulphoxide (25 ml) (V E 1 (b)) with gentle warming. The solution was then added, via a funnel inserted in the place of stopper 1, against a counter flow of dry nitrogen gas, to the reaction vessel. The temperature was raised to 50°C and the reaction mixture became brown in colour and a gel was formed. The reaction mixture was then stirred at room temperature for 15 h. The flask was cooled in ice water until the contents froze and methyl iodide (0.6 ml) (V E 1 (c)) was added dropwise. The stirring was continued at 20 – 25°C for 15 h.
Fig. VI 5 The apparatus for the further methylation of GS-5 R polysaccharide.

- CaCl₂ guard tube
- Stopper 2
- Stopper 1
- Dry nitrogen line
- Nitrogen cylinder and bubble counter (glycerol) and molecular sieve (4Å) tower and P₂O₅ tube and vent
- Vacuum supply
- 2 Liquid air traps to an Edwards high vacuum pump
- Heating and stirring block
The contents of the reaction vessel were poured into distilled water (50 ml) and the product was dialysed against distilled water (3 l) for 12 h. The dialysis water was concentrated to 10 ml in vacuo and the carbohydrate content of the concentrate was determined by the phenol-sulphuric acid method (V D 2). The concentrated dialysis water was subjected to paper chromatography in solvent (a).

The methylated product was dialysed against running tap water for 48 h, dialysed against distilled water for 48 h and extracted with cold chloroform (3 x 50 ml). The chloroform extracts were evaporated to dryness, suspended in water and freeze-dried. The methoxyl content of the product was determined and the infrared spectrum in the region 4000 cm\(^{-1}\) - 2500 cm\(^{-1}\) was obtained.

The whole reaction was repeated three times, omitting the extraction steps, and at the end of each methylation cycle any excess methyl iodide was removed by evacuating the system and pumping off the iodomethane. The final methylation was terminated by the addition of methyl iodide (6 ml).

The infrared spectrum of the final product in the region 4000 cm\(^{-1}\) - 2500 cm\(^{-1}\) was obtained using a Perkin Elmer P.E. 325 spectrophotometer with a dry atmosphere circulation system. The samples were prepared as potassium chloride discs.

The fate of the methylation product at each step in the methylation process and the relevant methoxyl contents are shown in figure VI 6. The infrared spectra of the products of each methylation step are shown in figure VI 7.
**Figure VI 6** The methylation of GS-5 B polysaccharide – Fate of the methylated product

GS-5 B polysaccharide

1st methylation

- Initial methylation VI A 7(a)
  - 450 mg (methoxyl content = 0%)
  - 400 mg
  - 370 mg (methoxyl content = 36%)
  - 230 mg

2nd methylation

- 200 mg (methoxyl content = 38.4%)

3rd methylation

- 130 mg

4th methylation

- 80 mg
Fig. VI 7 The infrared spectrum in the 4000 cm⁻¹-2500 cm⁻¹ region of the products of the various stages of the methylation of GS-5 B polysaccharide.

- Native polysaccharide
- 1st methylation
- 2nd methylation
- 3rd methylation
- 4th methylation
VI A 8 Hydrolysis of methylated GS-5 B polysaccharide

Six samples of methylated GS-5 B polysaccharide were hydrolysed VE 8 (a) for periods of 6, 7.5, 8.5, 10, 12 and 24 hours.

VI A 9 Reduction of hydrolysed, methylated GS-5 B polysaccharide

The hydrolysed, methylated GS-5 B polysaccharide samples were reduced as described (VE 5).

VI A 10 Acetylation of reduced, hydrolysed, methylated GS-5 B polysaccharide

The reduced, hydrolysed, methylated GS-5 B polysaccharide samples were acetylated as described (VE 9).

VI A 11 Analysis of acetylated, reduced, hydrolysed, methylated GS-5 B polysaccharide

The acetylated, reduced, hydrolysed, methylated GS-5 B polysaccharide samples were subjected to gas-liquid chromatography on a modified Perkin Elmer-Hitachi F11 RMS4 system (see Chapter VII).

The conditions employed were as follows:

Gas-liquid chromatography was performed using glass columns (2M x 1 mm) packed with packing material (i) (VA 5(a)). The carrier gas was dry helium and oven temperatures of 130-220°C were employed.

The all-glass interface system consisted of a Watson Bieman separator (separator temperature = 210°C) and a heated pipe inlet system (inlet system temperature = 220°C).

The mass spectrometer was operated at an ionising potential of 70 eV, with a slit width of 2 mm, a chamber temperature of 230-250°C and a scan speed of 3.
The mass spectra were recorded on direct print-out photographic paper. The relative areas under the peaks on the gas-liquid chromatography trace (g.l.c. trace) were determined by transferring the peaks onto constant density paper, cutting and weighing. The results are illustrated in table VI 7 and figure VI 8. The chromatogram of the 10 h hydrolysis is shown in figure VI 9 and the retention times obtained from this chromatogram are given in table VI 8. The mass spectra corresponding to the four major peaks in the chromatogram are shown in figures VI 10 – VI 13.

VI A 12 The partial acid hydrolysis of the GS-5 B polysaccharide

(a) The partial acid hydrolysis of GS-5 B polysaccharide

GS-5 B polysaccharide (100 mg) was hydrolysed with 0.5 M sulphuric acid (10 ml) for 1.5 h at 100°C on a steam bath. A standard dextran (Dextran T40, Sigma Biochemicals Ltd.) was simultaneously hydrolysed in a similar manner.

The hydrolysate was cooled, neutralised with barium carbonate and filtered. The resulting solution was deionised with IR 120(H⁺) and IRA 400 (OH⁻) resins, sequentially, and was evaporated to dryness.

Half the product was dissolved in the minimum volume of distilled water and was subjected to descending paper chromatography in solvent (b).

The remainder of the product was dissolved in distilled water (5 ml) and reduced using the borohydride reagents (VE 5). Following treatment with IR 120 (H⁺) resin, codistillation with methanol and evaporation to dryness, the reduced product was dissolved in the minimum volume of water and was subjected to descending paper
Table VI 7  The molar proportions of the various methylated glucitol derivatives as a function of hydrolysis time

<table>
<thead>
<tr>
<th>Hydrolysis time (h)</th>
<th>6</th>
<th>7.5</th>
<th>8.5</th>
<th>10</th>
<th>12</th>
<th>24</th>
</tr>
</thead>
<tbody>
<tr>
<td>Molar proportion of tetra-$\alpha$-methyl-$D$-glucitol derivative (%)</td>
<td>29</td>
<td>16.9</td>
<td>17.75</td>
<td>17.7</td>
<td>18.3</td>
<td>16.8</td>
</tr>
<tr>
<td>Molar proportion of tri-$\alpha$-methyl-$D$-glucitol derivative (%)</td>
<td>71</td>
<td>66.8</td>
<td>64.6</td>
<td>65.7</td>
<td>64.5</td>
<td>62.5</td>
</tr>
<tr>
<td>Molar proportion of di-$\alpha$-methyl-$D$-glucitol derivative (%)</td>
<td>0</td>
<td>16.3</td>
<td>17.7</td>
<td>16.6</td>
<td>17.2</td>
<td>20.7</td>
</tr>
</tbody>
</table>
Fig. VI 8 The molar proportions of the various methylated glucitol derivatives as a function of hydrolysis time.

- O O tri-O-methyl glucitol derivative
- □ □ tetra-O-methyl glucitol derivative
- ● ● di-O-methyl glucitol derivative

<table>
<thead>
<tr>
<th>molar proportion</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td></td>
</tr>
<tr>
<td>50</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>time (hours)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
</tr>
<tr>
<td>10</td>
</tr>
<tr>
<td>15</td>
</tr>
<tr>
<td>20</td>
</tr>
</tbody>
</table>
Fig. VI-9 The g.l.c. trace of the 10 hour hydrolysis of CS-5 B polysaccharide
<table>
<thead>
<tr>
<th>Component</th>
<th>Relative retention time $T_{\text{TMG}}^*$</th>
<th>Possible Identity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.00</td>
<td>1,5-di-_acetyl-2,3,4,6-tetra-_methyl-_D-glucitol</td>
</tr>
<tr>
<td>2</td>
<td>1.70</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>2.08</td>
<td>1,5,6-tri-_acetyl-2,3,4-tri-_methyl-_D-glucitol</td>
</tr>
<tr>
<td>4</td>
<td>3.76</td>
<td>1,3,5,6-tetra-_acetyl-2,4-di-_methyl-_D-glucitol</td>
</tr>
</tbody>
</table>

* $T_{\text{TMG}} = \text{Retention time relative to} \ 1,5$-di-\_acetyl-2,3,4,6-tetra-\_methyl-\_D-glucitol (TMG)
Fig. VI 10 Mass spectrum of component 1 of the acetylated, reduced 10 hr. hydrolysate of the methylated GS-5 B polysaccharide
Fig. VI 11 Mass spectrum of component 2
Fig. VI.13 Mass spectrum of component 4

m/e

189

129

117

43

% of base peak

100
chromatography in solvents (a) and (b) and paper electrophoresis in buffer (b). Staining reagent (a) was employed throughout.

The results are shown in tables VI 9 – VI 13.

(b) The separation of the products of partial hydrolysis of GS-5 B polysaccharide

The product of the partial hydrolysis of GS-5 B polysaccharide was subjected to preparative paper chromatography on Whatman No. 3 paper in solvent (b) (VA 1). Three components, compound 1, compound 2, and compound 3 were eluted from the paper (VA 1) and these compounds were rechromatographed in solvent (a). Each compound gave only a single spot on rechromatography.

(c) The characterisation of Compound 1

Component 1 was treated with activated charcoal and evaporated to a syrup.

The degree of polymerisation of compound 1 was determined as described (V D 7).

Preparation of the β-pentaacetate of Compound 1

Freshly fused sodium acetate (15 mg) was mixed with compound 1 (approx 10 mg) and refluxed with acetic anhydride (0.25 ml) for 40 minutes. The reaction mixture was poured onto crushed ice (1 ml) and the solution neutralised (pH 6.0) with sodium bicarbonate. The precipitated acetate was filtered, washed with distilled water, charcoalred from ethanol and recrystallised from aqueous ethanol. The melting point of the crystalline product and the mixed melting point with an authentic sample of glucose β-pentaacetate, was determined (table II 7).
### Table VI 9  
The partial hydrolysis of GS-5 B polysaccharide -  
Paper chromatography and degrees of polymerisation of the products  

<table>
<thead>
<tr>
<th>Chromatography solvent (b)</th>
<th>Staining reagent (a)</th>
<th>GS-5 B polysaccharide</th>
<th>Standard dextran*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>degree of polymerisation (estimated)</td>
<td>degree of polymerisation (estimated)</td>
</tr>
<tr>
<td>GS-5 B polysaccharide</td>
<td></td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>1.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3</td>
<td>2.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5</td>
<td>–</td>
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<td></td>
<td></td>
<td>6</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td></td>
<td>7</td>
<td>–</td>
</tr>
</tbody>
</table>

### Table VI 10  
The partial hydrolysis of GS-5 B polysaccharide -  
Paper chromatography of the reduced products  

<table>
<thead>
<tr>
<th>Chromatography solvent (a)</th>
<th>Staining reagent (a)</th>
<th>GS-5 B polysaccharide</th>
<th>Standard dextran*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>degree of polymerisation (estimated)</td>
<td>$E_G$</td>
</tr>
<tr>
<td>GS-5 B polysaccharide</td>
<td></td>
<td>1</td>
<td>0.91</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>0.55</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3</td>
<td>0.30</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4</td>
<td>0.16</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5</td>
<td>0.08</td>
</tr>
</tbody>
</table>

* Dextran T40 (Sigma Biochemicals Ltd.)
### Table VI 11  The partial hydrolysis of GS-5 B polysaccharide -
**Paper chromatography of the reduced products**

<table>
<thead>
<tr>
<th>Chromatography solvent (b)</th>
<th>Staining reagent (a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GS-5 B polysaccharide</td>
<td>Standard dextran*</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>degree of polymerisation (estimated)</th>
<th>$R_G$</th>
<th>$R_F$</th>
<th>$R_M$</th>
<th>$R_E$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.90</td>
<td>0.27</td>
<td>0.43</td>
<td>0.93</td>
</tr>
<tr>
<td>2</td>
<td>0.51</td>
<td>0.15</td>
<td>0.74</td>
<td>0.52</td>
</tr>
<tr>
<td>3</td>
<td>0.29</td>
<td>0.08</td>
<td>1.02</td>
<td>0.30</td>
</tr>
<tr>
<td>4</td>
<td>0.18</td>
<td>0.05</td>
<td>1.24</td>
<td>0.18</td>
</tr>
<tr>
<td>5</td>
<td>0.11</td>
<td>0.03</td>
<td>1.47</td>
<td>0.11</td>
</tr>
<tr>
<td>6</td>
<td>0.07</td>
<td>0.02</td>
<td>1.67</td>
<td>0.07</td>
</tr>
</tbody>
</table>

### Table VI 12  The partial hydrolysis of GS-5 B polysaccharide -
**Paper electrophoresis of the reduced products - Paper 1**

<table>
<thead>
<tr>
<th>Electrophoresis buffer (b)</th>
<th>Staining reagent (a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GS-5 B polysaccharide</td>
<td>Standard dextran*</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>degree of polymerisation (estimated)</th>
<th>$M_S$</th>
<th>$1/M_S$</th>
<th>$M_S$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>2</td>
<td>0.78</td>
<td>1.28</td>
<td>0.79</td>
</tr>
<tr>
<td>3</td>
<td>0.63</td>
<td>1.59</td>
<td>0.63</td>
</tr>
<tr>
<td>4</td>
<td>0.54</td>
<td>1.85</td>
<td>0.54</td>
</tr>
<tr>
<td>5</td>
<td>0.47</td>
<td>2.13</td>
<td>0.47</td>
</tr>
<tr>
<td>6</td>
<td>0.42</td>
<td>2.38</td>
<td>0.42</td>
</tr>
<tr>
<td>7</td>
<td>0.39</td>
<td>2.56</td>
<td>0.39</td>
</tr>
<tr>
<td>8</td>
<td>0.36</td>
<td>2.78</td>
<td>0.36</td>
</tr>
</tbody>
</table>

* Dextran T40 (Sigma Biochemicals Ltd.)
Table VI 13 The partial hydrolysis of GS-5 B polysaccharide

<table>
<thead>
<tr>
<th>Degree of polymerisation (estimated)</th>
<th>(M_S)</th>
<th>(1/M_S)</th>
<th>(M_S)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.01</td>
<td>0.99</td>
<td>0.99</td>
</tr>
<tr>
<td>2</td>
<td>0.74</td>
<td>1.35</td>
<td>0.74</td>
</tr>
<tr>
<td>3</td>
<td>0.60</td>
<td>1.67</td>
<td>0.60</td>
</tr>
<tr>
<td>4</td>
<td>0.51</td>
<td>1.96</td>
<td>0.51</td>
</tr>
<tr>
<td>5</td>
<td>0.45</td>
<td>2.22</td>
<td>0.45</td>
</tr>
<tr>
<td>6</td>
<td>0.41</td>
<td>2.44</td>
<td>0.41</td>
</tr>
</tbody>
</table>

* Dextran T40 (Sigma Biochemicals Ltd.)

(d) The characterisation of Compound 2

Compound 2 was treated with activated charcoal and evaporated to a syrup.

The degree of polymerisation of compound 2 was determined as described (V D 7).

Preparation of the \(\beta\)-octaacetate of Compound 2

Freshly fused sodium acetate (15 mg) was mixed with compound 2 (approx. 10 mg) and refluxed with acetic anhydride (0.25 ml) for 1 h. The reaction mixture was poured onto crushed ice (1 ml) and the solution was neutralised (pH 6.0) with sodium bicarbonate. The precipitated acetate was filtered, washed with distilled water, charcoaled from ethanol and recrystallised from aqueous ethanol. The melting point of the crystalline product and the mixed melting point
with an authentic sample of isomaltose β-octaacetate were determined (table II 7).

(e) The characterisation of Compound 3

Compound 3 was treated with activated charcoal and evaporated to a syrup.

The degree of polymerisation of compound 3 was determined as described (V D 7) (table II 7).

VI A 13 The partial acetylation of GS-5 B polysaccharide

(a) The partial acetylation of GS-5 B polysaccharide

GS-5 B polysaccharide (150 mg) and a standard of glucose A.R. (150 mg) were treated with acetic anhydride:conc. sulphuric acid (100:9 v:v) (1.2 ml) with cooling in an ice-bath. The acetylation mixtures were kept at 35-37°C for 32 h in capped tubes, with occasional shaking to assist complete solution. The mixed acetates were poured into ice-cold water (20 ml), neutralised to pH 7.1 - 7.3 with sodium bicarbonate, and left to stand overnight and the acetates were then extracted with chloroform (3 x 20 ml). The chloroform extracts were washed with saturated sodium bicarbonate solution and distilled water and were then dried for 24 h over anhydrous sodium sulphate. The solutions were filtered and evaporated to dryness.

Deacetylation was achieved with sodium metal and 'superdry' methanol (V E 1 (d)). The solid residues were dried over conc. sulphuric acid in a desiccator for 24 hr and were treated with a small piece of clean sodium metal in 'superdry' methanol (2 ml). The reaction was left at 4°C for 24 h. The white precipitates that
formed were redissolved in distilled water, (5 ml) which was added to each flask. The sodium ions were removed by shaking with IR 120 (H⁺) resin for 24 h. The solutions were filtered and evaporated to dryness to remove any remaining acetic acid. The free sugars were subjected to paper chromatography in solvents (a) and (b). The results are shown in tables VI 14 and VI 15.

(b) The separation of the products of acetolysis of GS-5 B polysaccharide

The product of the partial acetolysis of GS-5 B polysaccharide was subjected to preparative paper chromatography on Whatman No. 3 paper in solvent (b). The components of the acetolysate were eluted from the paper (VI A 1) and were rechromatographed in solvent (c). Staining reagent (a) was employed throughout.

(c) The characterisation of Compound 12

Compound 12 was treated with activated charcoal and evaporated to a syrup.

Part of the product was dissolved in the minimum volume of distilled water and was subjected to electrophoresis in buffer (a).

A further portion of the product was dissolved in distilled water (5 ml) and was reduced using the borohydride reagents (V E 5). After treatment with IR 120 (H⁺) resin, codistillation with methanol and evaporation to dryness, the reduced compound 2 was subjected to electrophoresis in buffer (b).

The degree of polymerisation of compound 12 was determined as described (V D 7).
### Table VI 14
The partial acetylolation of GS-5 B polysaccharide - Paper chromatography and degrees of polymerisation of the products

<table>
<thead>
<tr>
<th>Compound</th>
<th>Acetolysate of GS-5 B polysaccharide</th>
<th>Partial hydrolysate degree of dextran**</th>
<th>Estimated degree of standard polymerisation</th>
<th>Possible identity of compound</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$E_g$</td>
<td>$E_g$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>0.99</td>
<td>1.00</td>
<td>1</td>
<td>glucose</td>
</tr>
<tr>
<td>12</td>
<td>0.76</td>
<td>1.9*</td>
<td>2</td>
<td>disaccharide</td>
</tr>
<tr>
<td>13</td>
<td>0.53</td>
<td>0.53</td>
<td>2</td>
<td>isomaltose</td>
</tr>
<tr>
<td>14</td>
<td>0.38</td>
<td>3</td>
<td>3</td>
<td>branch trisaccharide</td>
</tr>
<tr>
<td>15</td>
<td>0.26</td>
<td>0.26</td>
<td>3</td>
<td>isomaltotriose</td>
</tr>
<tr>
<td>16</td>
<td>0.19</td>
<td>4</td>
<td>4</td>
<td>branch tetrasaccharide</td>
</tr>
<tr>
<td>17</td>
<td>0.12</td>
<td>0.12</td>
<td>4</td>
<td>isomaltotetraose</td>
</tr>
</tbody>
</table>

All spots were grey-green in colour when the developed papers are sprayed with staining reagent (c).

### Table VI 15
The partial acetylolation of GS-5 B polysaccharide - Paper chromatography and degrees of polymerisation of the products

<table>
<thead>
<tr>
<th>Compound</th>
<th>Acetolysate of GS-5 B polysaccharide</th>
<th>Partial hydrolysate degree of dextran**</th>
<th>Estimated degree of standard polymerisation</th>
<th>Possible identity of compound</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$E_g$</td>
<td>$E_g$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>0.96</td>
<td>1.02</td>
<td>1</td>
<td>glucose</td>
</tr>
<tr>
<td>12</td>
<td>0.71</td>
<td>2</td>
<td>2</td>
<td>disaccharide</td>
</tr>
<tr>
<td>13</td>
<td>0.52</td>
<td>0.56</td>
<td>2</td>
<td>isomaltose</td>
</tr>
<tr>
<td>14</td>
<td>0.37</td>
<td>3</td>
<td>3</td>
<td>branch trisaccharide</td>
</tr>
<tr>
<td>15</td>
<td>0.30</td>
<td>0.32</td>
<td>3</td>
<td>isomaltotriose</td>
</tr>
<tr>
<td>16</td>
<td>0.25</td>
<td>4</td>
<td>4</td>
<td>branch tetrasaccharide</td>
</tr>
<tr>
<td>17</td>
<td>0.19</td>
<td>4</td>
<td>4</td>
<td>isomaltotetraose</td>
</tr>
</tbody>
</table>

* determined by method (V D 7) ** Dextran T40 Sigma Biochemicals Ltd.
Preparation of the β-octaacetate of Compound 12

Freshly fused sodium acetate (15 mg) was mixed with compound 12 (approx. 3 mg) and refluxed with acetic anhydride (0.25 ml) for 1 h. The reaction mixture was poured onto crushed ice (1 ml) and the solution was neutralised (pH 6.0) with sodium bicarbonate. The precipitated acetate was filtered, washed with distilled water, charcoaled from ethanol and recrystallised from aqueous ethanol. The melting point of the crystalline product was determined (table II 11). No material remained for a mixed melting point determination. Some properties of compound 12 and the β-octaacetate of component 12 are shown in tables II 10 and II 11.

VI A 14 The enzymic hydrolysis of GS-5 B polysaccharide

(a) Qualitative digests

(i) Small scale qualitative digests

The endodextranase of P. lilacinum was prepared as described (V E 10).

Qualitative digests were made up as follows:—

P. lilacinum dextranase 2 ml (1 mg of freeze-dried enzyme).
Sodium citrate buffer pH 5.0 (buffer B1) 2 ml
Polysaccharide 5 mg.

Digests containing GS-5 B polysaccharide, S. bovis strain I dextran and a reagent blank containing enzyme and buffer only were prepared.

The digests were sealed with a layer of toluene A.R. to discourage microbial and mould growth and were incubated at 37°C for 48 h.
After incubation, the digests were deionised with IR 120 (H⁺) and IRA 400 (OH⁻) resins sequentially, filtered, and the solutions were evaporated to a small volume and subjected to paper chromatography in solvent (a).

The results are shown in tables VI 16 and VI 17.

Table VI 16 The P. lilacinum dextranase digest of GS-5 B polysaccharide - Paper chromatography of the enzymic hydrolysate

<table>
<thead>
<tr>
<th>Compound</th>
<th>Rf</th>
<th>Intensity by visual inspection</th>
<th>Possible identity</th>
</tr>
</thead>
<tbody>
<tr>
<td>21</td>
<td>1.01</td>
<td>+</td>
<td>glucose</td>
</tr>
<tr>
<td>22</td>
<td>0.54</td>
<td>3+</td>
<td>isomaltose</td>
</tr>
<tr>
<td>23</td>
<td>0.27</td>
<td>+</td>
<td>isomaltotriose</td>
</tr>
<tr>
<td>24</td>
<td>0.081</td>
<td>+</td>
<td>'branch' pentasaccharide*</td>
</tr>
<tr>
<td>25</td>
<td>0.038</td>
<td>+</td>
<td>'branch' hexasaccharide*</td>
</tr>
<tr>
<td>26</td>
<td>0.020</td>
<td>+</td>
<td>'branch' heptasaccharide*</td>
</tr>
<tr>
<td>27</td>
<td>unresolved material</td>
<td>+</td>
<td>unresolved material</td>
</tr>
</tbody>
</table>

* A 'branch' oligosaccharide in this context is defined as an oligosaccharide containing a secondary [i.e. non-α-(1→6)] glucosidic linkage.

The components of the hydrolysate were identified by comparison with standard compounds.
Table VI 17  The *P. lilacinum* dextranase digest of *S. bovis*

Strain 1 dextran - Paper chromatography of the enzymic hydrolysate

<table>
<thead>
<tr>
<th>Compound</th>
<th>$R_g$</th>
<th>Intensity by visual inspection</th>
<th>Possible identity</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>1.02</td>
<td>+</td>
<td>glucose</td>
</tr>
<tr>
<td>B</td>
<td>0.55</td>
<td>4+</td>
<td>isomaltose</td>
</tr>
<tr>
<td>C</td>
<td>0.28</td>
<td>2+</td>
<td>isomaltotriose</td>
</tr>
<tr>
<td>D</td>
<td>0.080</td>
<td>trace</td>
<td>'branch' pentasaccharide*</td>
</tr>
<tr>
<td>E</td>
<td>0.039</td>
<td>trace</td>
<td>'branch' hexasaccharide*</td>
</tr>
<tr>
<td>F</td>
<td>unresolved material</td>
<td>+</td>
<td>unresolved material</td>
</tr>
</tbody>
</table>

(ii) Large scale qualitative digest

A large scale qualitative digest of GS-5 B polysaccharide with *P. lilacinum* dextranase was prepared as follows.

*P. lilacinum* dextranase 100 mg of freeze-dried enzyme.

Sodium citrate buffer pH 5.0 (buffer B1)

GS-5 B polysaccharide 500 mg.

The digest was sealed with a layer of toluene A.R. and was incubated at 37°C for 48 h.

After incubation, the digests were deionised with IR 120 ($H^+$) and IRA 400 ($OH^-$) resins sequentially, filtered, and the solutions were evaporated to a small volume and subjected to preparative paper chromatography on Whatman No.3 paper in solvent (c) for 4 days.
The resulting compounds were eluted from the paper, rechromatographed in solvent (c) evaporated to dryness and the yields were recorded. The individual compounds were then subjected to paper chromatography in solvent (c). The results are shown in table VI 18.

Table VI 18 The *P. lilacinum* dextranase digest of GS-5 B polysaccharide - Preparative paper chromatography of the products of a large scale digest - Yields and \( R_{\text{M}_5} \) values

<table>
<thead>
<tr>
<th>Chromatography solvent (c)</th>
<th>Staining reagent (a)</th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th>Compound</th>
<th>Degree of polymerisation (estimated)</th>
<th>Yield from 500 mg of polysaccharide (mg)</th>
<th>( R_{\text{M}_5} )</th>
<th>( R_P )</th>
<th>( R_M )</th>
<th>Possible identity</th>
</tr>
</thead>
<tbody>
<tr>
<td>24</td>
<td>5</td>
<td>30.0</td>
<td>1.25</td>
<td>0.03</td>
<td>1.510</td>
<td>'branch' penta-saccharide</td>
</tr>
<tr>
<td>25</td>
<td>6</td>
<td>45.7</td>
<td>0.84</td>
<td>0.02</td>
<td>1.690</td>
<td>'branch' hexa-saccharide</td>
</tr>
<tr>
<td>26</td>
<td>7</td>
<td>25.1</td>
<td>0.53</td>
<td>0.012</td>
<td>1.891</td>
<td>'branch' hepta-saccharide</td>
</tr>
<tr>
<td>27</td>
<td>8</td>
<td>15.4</td>
<td>0.40</td>
<td>0.0096</td>
<td>2.014</td>
<td>'branch' octa-saccharide</td>
</tr>
<tr>
<td>28</td>
<td>9</td>
<td>15.4</td>
<td>0.30</td>
<td>0.0072</td>
<td>2.440</td>
<td>'branch' nono-saccharide</td>
</tr>
<tr>
<td>29</td>
<td>&gt;9</td>
<td>≤ 0.20</td>
<td></td>
<td></td>
<td></td>
<td>unresolved material</td>
</tr>
</tbody>
</table>

Since the glucose standard was eluted from the paper it was necessary to use isomaltopentaose as a standard. The \( R_P \) values were determined by extrapolation.

(b) **Quantitative digest**

A standard solution containing *P. lilacinum* dextranase (freeze-dried) (21.5 mg) in sodium citrate buffer pH 5.0 (buffer B1) (30 ml) was prepared.
This was used immediately after preparation for quantitative
P. lilacinum dextranase digests of all the polysaccharides so treated.

Digests were prepared as follows:

The above solution 3 ml
Polysaccharide (vacuum-dried over phosphorous pentoxide for 3 days) 10 mg

The digests were sealed with a layer of toluene A.R. to discourage
microbial and mould growth and were incubated at 37°C for 48 h.

Digests of GS-5 B polysaccharide and of a standard dextran T40
were prepared.

Aliquots (100 μl) were taken periodically and subjected to the
Nelson test for reducing sugars (V D 3).

VI A 15 Gel permeation chromatography of GS-5 B polysaccharide

(a) Gel permeation chromatography on Sepharose 6B,*

Sephrose 2B, * Sephadex G-200* and Sephadex G-100*

The Sepharose gels were prepared as described in VA 6(a)
and the Sephadex gels as described in VA 6(b).

(i) Sepharose 2B

A K15/30 column (Pharmacia G.B.) was packed with
prepared gel as described (VA 6 (a)). The eluant employed was 1%
sodium chloride solution and a working pressure of 3-6 cm of 1%
sodium chloride solution was employed adjusted to give a flow rate of
0.05 ml per min. Loadings of Blue dextran 2000* (10 mg), glucose
(10 mg) and GS-5 B polysaccharide (25 mg) each dissolved in 1%
sodium chloride solution (1 ml) were applied to the column. 2.0 ml fractions
were collected.

The blue dextran was estimated by directly reading the solution
at 600 nm on a SP 500 spectrophotometer. The glucose and
polysaccharide were estimated as follows. An aliquot (200 μl) was taken from each fraction and distilled water (800 μl) was added. The solution was then estimated by the phenol-sulphuric acid method (V D 2).

(ii) Sepharose 6B

A K15/90 column (Pharmacia G.B.) was packed with prepared gel as described (VA 6(a)). The eluant employed was 1% sodium chloride solution and a working pressure of 3–6 cm of 1% sodium chloride solution was employed adjusted to give a flow rate of 0.05 ml/min. Loadings of Blue Dextran 2000* (10 mg) Dextran T10**(\(\bar{M}_w = 10,000\)), Soluble laminarin*** (\(\bar{M}_w = 2000\)) (25 mg) GS-5 B polysaccharide (25 mg) and glucose (10 mg) each dissolved in 1% sodium chloride solution (1 ml) were applied to the column, 2.0 ml fractions were collected.

Blue dextran and glucose and polysaccharides were estimated as in VI A 15(a)(i).

(iii) Sephadex G-100

A K15/90 column (Pharmacia G.B.) was packed with prepared gel as described (VA 6(b)). The eluant employed was 1% sodium chloride solution and a working pressure of 30 cm of 1% sodium chloride solution was employed giving a flow rate of 0.5 ml per minute.

* Obtained from Pharmacia (G.B.) Ltd.
** Kindly donated by Dr. J. John Marshall, Royal Holloway College.
*** Obtained from the departmental collection.
Loadings of Blue Dextran 2000\(^*\) (10 mg), GS-5 B polysaccharide (25 mg) and glucose (10 mg) each dissolved in 1% sodium chloride solution (1 ml) were applied to the column. 2.0 ml fractions were collected.

Blue dextran and glucose and polysaccharide were estimated as in VI A 15 (a) (i).

(iv) Sephadex G-200

A K15/90 column (Pharmacia G.B.) was packed with prepared gel as described (VA 6 (b)). The eluant employed was 1% sodium chloride solution and a working pressure of 13 cm of 1% sodium chloride solution was employed giving a flow rate of 0.1 ml per minute.

Loadings of Blue dextran 2000\(^*\) (10 mg) GS-5B polysaccharide (25 mg) and glucose (10 mg) dissolved in 1% sodium chloride solution (1 ml) were applied to the column. 2.0 ml fractions were collected.

Blue dextran, glucose and GS-5 B polysaccharide were estimated as in VI A 15(a)(i). The results are shown in table VI 19 and figure II 25.

(b) Calibration of the Sephadex G-200 column

The Sephadex G-200 column was calibrated using:

- **Blue Dextran 2000\(^*\)** \(\bar{M}_W = 2 \times 10^6\) (10 mg) in 1% sodium chloride solution (1 ml).
- **Betacoccus dextran\(^***\)** \(\bar{M}_W = 7.5 \times 10^4\) (25 mg) in 1% sodium chloride solution (1 ml).
- **Dextran T40\(^*\)** \(\bar{M}_W = 4 \times 10^4\) (25 mg) in 1% sodium chloride solution (1 ml).
- **Dextran 10\(^*\)** \(\bar{M}_W = 1 \times 10^4\) (25 mg) in 1% sodium chloride solution (1 ml).
- **Glucose A.R.** \(\bar{M}_W = 1\) (10 mg) in 1% sodium chloride solution (1 ml).
and the elution diagrams of these materials on Sephadex G-200 are shown in figure VI 14. A plot of log $\bar{M}_w$ versus $K_{av}$ where $K_{av}$ is the partition coefficient between the liquid phase and the gel phase for a molecule having a molecular weight equal to $\bar{M}_w$, and $K_{av}$ is given by the equation

$$K_{av} = \frac{Ve-Vo}{Vt-Vo}$$

$Ve$ = elution volume of a molecular species having a molecular weight equal to $\bar{M}_w$.

$Vo$ = the void volume.

$Vt$ = the total volume of the bed

is shown in figure VI 15.

The standards were estimated as described in VI a 15(a) (i).

The intensities of the colours were read on an Eel spectrophotometer and are quoted as percentage scale deflections.

(c) Confirmation of the molecular distribution of GS-5 B polysaccharide by ultracentrifugation

GS-5 B polysaccharide (50 mg) in 0.1M potassium chloride solution (5 ml) was subjected to ultracentrifugation in a Spinco Model E Ultracentrifuge at 59,780 rev/min for 5 h at 20°C.

The image was photographed at intervals and the results are illustrated in figure II 26.

(d) Preparative gel permeation chromatography of GS-5 B polysaccharide on Sephadex G-200

A K26/100 column [Pharmacia (G.B.)] was packed with prepared gel as described (VA 6 (b)). The eluant employed was 1% sodium chloride solution. The working head was adjusted (10 cm-20 cm) to
Fig. V.14 Calibration of Sephadex G-200 column. Elution diagram of standards.
Fig. VI.15 Calibration of Sephadex G-200 column $k_{av}$ versus log $M_w$. 

$\bar{M}_w = 10,000$

$\bar{M}_w = 40,000$

$\bar{M}_w = 75,000$
give a flow rate of 0.1 ml per min. Loadings of Blue dextran 2000
(25 mg) in 1% sodium chloride (3 ml), glucose (60 mg) in 1% sodium
chloride (2 ml) and GS-5 B polysaccharide (2 x 250 mg) in 1% sodium
chloride (3 ml) were applied to the column. 5.0 ml fractions were
taken.

The results are illustrated in figure II 27.

The GS-5 B polysaccharide and the standards were estimated as in
VI A 15(a)(i).

The contents of tubes 35-65 were combined. The product was
freeze-dried, dried in vacuo over phosphorous pentoxide and weighed.
This was designated Fraction I.

The contents of tubes 65-100 were combined. The product, treated as
above, was designated Fraction II.

The fructose contents of Fractions I and II were determined
(V D 5) and are shown in table II 12.

Enzymic digests were prepared as follows:
GS-5 B polysaccharide Fraction I or II 5 mg
E. lilacinum dextranase 2 ml
Sodium citrate buffer pH 5.0 (buffer B1) 2 ml. A reagent blank
containing the enzyme and the buffer only was prepared.

The digests were sealed with a layer of toluene A.R. to
discourage microbial and mould growth and were incubated at 37°C
for 48 h.

After incubation the digests were deionised with IR 120 (H+ )
and IRA 400 (OH−) resins sequentially, filtered, and the solutions
were evaporated to a small volume and subjected to paper chromatography
in solvent (a).
Table VI 19  Gel permeation chromatography of GS-5 B polysaccharide
(Figure 11.25)

<table>
<thead>
<tr>
<th>Elution volume Ve (ml)</th>
<th>Absorbance</th>
</tr>
</thead>
<tbody>
<tr>
<td>40</td>
<td>0.210</td>
</tr>
<tr>
<td>42</td>
<td>0.340</td>
</tr>
<tr>
<td>44</td>
<td>0.485</td>
</tr>
<tr>
<td>46</td>
<td>0.612</td>
</tr>
<tr>
<td>48</td>
<td>0.788</td>
</tr>
<tr>
<td>50</td>
<td>0.955</td>
</tr>
<tr>
<td>52</td>
<td>1.04</td>
</tr>
<tr>
<td>54</td>
<td>1.00</td>
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<tr>
<td>56</td>
<td>1.09</td>
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<tr>
<td>58</td>
<td>1.18</td>
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<td>60</td>
<td>1.17</td>
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<td>62</td>
<td>1.11</td>
</tr>
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<td>64</td>
<td>1.12</td>
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<tr>
<td>66</td>
<td>1.12</td>
</tr>
<tr>
<td>68</td>
<td>1.12</td>
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<tr>
<td>70</td>
<td>1.05</td>
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<tr>
<td>72</td>
<td>1.03</td>
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<tr>
<td>74</td>
<td>0.940</td>
</tr>
<tr>
<td>76</td>
<td>0.902</td>
</tr>
<tr>
<td>78</td>
<td>0.920</td>
</tr>
<tr>
<td>80</td>
<td>0.879</td>
</tr>
<tr>
<td>82</td>
<td>0.840</td>
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<tr>
<td>84</td>
<td>0.772</td>
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<tr>
<td>86</td>
<td>0.762</td>
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<tr>
<td>88</td>
<td>0.800</td>
</tr>
<tr>
<td>90</td>
<td>0.733</td>
</tr>
<tr>
<td>92</td>
<td>1.01</td>
</tr>
<tr>
<td>94</td>
<td>0.820</td>
</tr>
<tr>
<td>96</td>
<td>0.795</td>
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</tbody>
</table>

/continued
<table>
<thead>
<tr>
<th>Elution volume Ve (ml)</th>
<th>Absorbance</th>
</tr>
</thead>
<tbody>
<tr>
<td>98</td>
<td>.775</td>
</tr>
<tr>
<td>100</td>
<td>.709</td>
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<tr>
<td>102</td>
<td>.650</td>
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<tr>
<td>104</td>
<td>.575</td>
</tr>
<tr>
<td>106</td>
<td>.453</td>
</tr>
<tr>
<td>108</td>
<td>.340</td>
</tr>
<tr>
<td>110</td>
<td>.235</td>
</tr>
<tr>
<td>112</td>
<td>.148</td>
</tr>
</tbody>
</table>

$V_o = 40 \text{ ml}$

$V_t = 124 \text{ ml}$

The results are shown in Table VI 20.

**Table VI 20**

Preparative gel permeation chromatography of GS-5 B polysaccharide - P. lilacinum dextranase digests of Fractions I and II - Paper chromatography of products

<table>
<thead>
<tr>
<th>Component</th>
<th>Fraction I</th>
<th>Fraction II</th>
<th>Possible Identity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Retention</td>
<td>Intensity</td>
<td>Retention</td>
</tr>
<tr>
<td>Compound 1</td>
<td>1.00</td>
<td>+</td>
<td>1.01</td>
</tr>
<tr>
<td>Compound 2</td>
<td>0.54</td>
<td>+++</td>
<td>0.54</td>
</tr>
<tr>
<td>Compound 3</td>
<td>0.27</td>
<td>+</td>
<td>0.27</td>
</tr>
<tr>
<td>Compound 4</td>
<td>0.081</td>
<td>+</td>
<td>0.081</td>
</tr>
<tr>
<td>Compound 5</td>
<td>0.035</td>
<td>+</td>
<td>0.035</td>
</tr>
<tr>
<td>Compound 6</td>
<td>0.018</td>
<td>+</td>
<td>0.019</td>
</tr>
<tr>
<td>Compound 7</td>
<td>0.0092</td>
<td>+</td>
<td>unresolved</td>
</tr>
<tr>
<td>Compound 8</td>
<td>unresolved</td>
<td>+</td>
<td></td>
</tr>
</tbody>
</table>
Fraction I, Fraction II, and a glucose blank (c. 10 mg) (dried in vacuo at 35°C for 2 days) were methylated as described (VE 7). The yields are shown in table VI 21.

The methylated products were hydrolysed (VE 8), reduced (VE 5) and acetylated (VE 9) and subjected to g.l.c. - m.s. analysis.

The g.l.c. traces obtained and the mass spectra of the components are shown in figures VI 18 to VI 25.

The results are tabulated in table II 13.

Table VI 21 The methylation of GS-5 B polysaccharide - the yields of the methylated products

<table>
<thead>
<tr>
<th>polysaccharide</th>
<th>Wt. of starting material (mg)</th>
<th>Wt. of product (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fraction I</td>
<td>11.0</td>
<td>10.0</td>
</tr>
<tr>
<td>Fraction II</td>
<td>10.0</td>
<td>10.3</td>
</tr>
</tbody>
</table>

(e) Gel permeation chromatography of dimethylsulphoxide-treated GS-5 B polysaccharide on Sephadex G-200

GS-5 B polysaccharide (50 mg) was dissolved in dimethylsulphoxide (5 ml) and heated at 70°C for 1 h. The reaction was then left to stand at room temperature for 24 h. The solution was dialysed against running tap water for 3 days and against distilled water for 3 days. The product was freeze-dried and subjected to gel permeation chromatography on the column used in VI A 15 (a). The elution diagram is shown, compared to untreated GS-5 B polysaccharide, in figure II 25.
Fig. VI 16  Gel permeation chromatography of GS-5 B polysaccharide on Sephadex G-200. g.l.c trace of product of methylation analysis of fraction 1
Fig. VI 17 Gel permeation chromatography of GS-5 B polysaccharide on Sephadex G-200. g.l.c. trace of product of methylated analysis of Fraction II
Fig. VI 18  Gel filtration of GS-5 B polysaccharide on Sephadex G200. Fraction I. Mass spectrum of component 1 of acetylated, reduced, hydrolysed, methylated GS-5 B polysaccharide.
Fig. VI 19  Fraction I. Mass spectrum of component 2
Fig. VI 20  Fraction I. Mass spectrum of component 3
Fig. VI 21 Gel filtration of GS-5 B polysaccharide on Sephadex G-200. Fraction II. Mass spectrum of component 1.
Fig. VI 22  Fraction II. Mass spectrum of component 2
Fig. VI.23 Fraction II. Mass spectrum of component 3
VI A 16 The mild hydrolysis of GS-5 B polysaccharide

(a) Initial hydrolysis

GS-5 B polysaccharide (25 mg) (exhaustively dried) was dissolved in 0.1M sulphuric acid (0.5 ml) in a 1 ml stoppered flask. The flask was capped, wired and heated on a water bath at 60°C.

Aliquots (10 μl) of the solution was taken at intervals and added to distilled water (100 μl) in stoppered tubes, cooled in ice water to stop evaporation. The contents of the tubes were mixed by gentle shaking.

To the diluted aliquots (10 μl) distilled water (1 ml) was added and the glucose contents were determined (V D 4 (a)).

To the diluted aliquots (50 μl) distilled water (1 ml) was added and the reducing sugar contents were determined (V D 3). A graph of reducing sugars and glucose released (mg) in the total digest against time was plotted. The results are shown in figure II 28.

(b) Preparative mild hydrolysis

GS-5 B polysaccharide (25 mg) (exhaustively dried) was dissolved in 0.1M sulphuric acid (0.5 ml) in a 1 ml stoppered flask. The flask was capped and heated on a water bath at 60°C.

After 75 h the reaction was terminated by neutralisation with barium carbonate. The solid material was removed by filtration.

The product (approx. 2. 15 mg) was subjected to gel permeation chromatography on Sephadex G-200 (V A 6 (b)) on a Pharmacia K16/100 column (figure VI 24).

A further sample of the product ([. 10 mg) was evaporated to dryness and subjected to methylation analysis as in VI A 15(d). The g.l.c. trace is illustrated in figure VI 25 and the mass spectra in figures VI 26 and VI 27.
Fig. VI 24 The mild acid hydrolysis of GS-5 B polysaccharide. Gel permeation chromatography of the mild hydrolysate
Fig. VI 25  The mild hydrolysis of GS-5 B polysaccharide. Methylation analysis of the product.  g.l.c. trace

187°C

absolute retention time (min)
Fig. VI 26  The mild hydrolysis of GS-5 B polysaccharide. Methylation analysis of the mild hydrolysate. Mass spectrum of component 1.
Fig. VI 27  Mass spectrum of component 2

% of base peak

43  45  87  99  101  117  129  161  189

m/e
VI A 17 Attempted preparation of an exodextranase from *Bacillus megatherium* strain D2

(a) Maintenance of *B. megatherium* strain D2

The organism was generously donated by Dr. L.P.T.N. Zevenhuisen, Wageningen, Nederlands. The lyophilised organism was initially grown on medium M4 at 30°C for 24 h. Stabbings were then transferred to nutrient broth (Oxoid) and the cultures were incubated at 30°C until the organisms were well established.

(b) Initial attempted preparation of the exodextranase from *B. megatherium*

An aliquot (1 ml) of the nutrient broth culture (VI A 17 (a)) was transferred to a sterile fermentation vessel containing medium M7 (1 l). The culture medium was aerated with sterile air for 5 days at 30°C. The cells were harvested by centrifugation at 2800 g at 0°C.

The cells were washed with distilled water, resuspended in distilled water (15 ml), and the cells were disintegrated using a M.S.E. ultrasonic disintegrator (micro-probe, setting medium). The cells were disintegrated for three one minute periods with one minute periods of cooling in ice-water between each disintegration.

The resulting material was freeze-dried and the freeze-dried product (25 mg) was dissolved in distilled water (3 ml).

Digests were prepared as follows:-

Buffer B2 0.8 ml

1% *Streptococcus bovis* strain I dextran in water 0.8 ml

Enzyme preparation 0.4 ml

The digest was incubated at 30°C for 24 h under a layer of toluene A.R. to discourage bacterial and mould growth.
An aliquot (0.5 ml) was subjected to the Nelson test for reducing sugars (V D 3) to detect enzyme activity.

(c) Modification of the attempted preparation of the exo-
dextranase from B. megatherium

An aliquot (1 ml) of the nutrient broth culture (VI A 17 (a)) was aseptically transferred to a cotton-wool-plugged 1 l Erlemeyer flask containing medium M8 (250 ml). The flasks were incubated at 30°C on an orbital incubator with orbital agitation. The concentration of bacteria was determined periodically by method V B 1 (d). The growth curve is illustrated in figure VI 28.

Further aliquots (1 ml) of the nutrient broth culture (VI A 17(a)) were aseptically transferred to 6 cotton-wool-plugged 1 l Erlemeyer flasks each containing medium M8 (250 ml). The experiment was stopped after 35 h (mid-way through the exponential growth period). Samples of the organism were cultured onto slopes of medium M4. These slopes were stored at 4°C for maintenance purposes. The remainder of the bacterial cells were harvested by centrifugation at 2800 g. The cells were resuspended in water (25 ml) and disintegrated using the M.S.E. ultrasonic disintegrator (micro-probe, setting medium). The cells were disintegrated for three one minute periods with one minute periods of cooling in ice-water between each disintegration.

The crude enzyme was centrifuged on the M.S.E. bench top centrifuge and the supernatant (the enzyme preparation) was decanted off and retained. The cell debris was examined microscopically after staining with stain S1.
Fig. VI 28. The growth curve of B. megatherium D₁ (VI A 17(c)).
Digests were prepared as follows:-

Buffer B2 0.4 ml

1% Streptococcus bovis strain I dextran 0.4 ml
in water

Enzyme preparation 0.2 ml

The digests were incubated at 30°C for 24 h under a layer of toluene A.R. to discourage bacterial and mould growth.

The product was subjected to the Nelson test for reducing sugars (V D 3) and to the glucose oxidase/peroxidase test for free glucose (V D 4 (c)). The product, following deionisation was subjected to paper chromatography in solvent (d), staining reagent (a) was employed.

(d) Further modification of the attempted preparation of the exodextranase from _B. megatherium_

The _B. megatherium_ organism was cultured as described in VI A 17(c). One of the cultures was set aside after 35 h and the remaining five cultures were stored in a refrigerator (4°C) until required. The cells were harvested by centrifugation 2800 g and the resulting bacterial cells (0.8 g) were suspended in buffer B4 (5 ml). The cells were disintegrated using a _M.S.E._ ultrasonic disintegrator (micro-probe, setting medium) for three one minute periods, six one minute periods and nine one minute periods, with periods of cooling as described in VI A 17 (c).

Digests of the three enzyme preparations were prepared as in VI A 17 (c) and assays were carried out as described.

The remaining five cultures were taken from store. The cells were harvested by centrifugation and the resulting bacterial cells (4 g) were suspended in buffer B4 (25 ml). The cells were disintegrated using a _M.S.E._ ultrasonic disintegrator (medium probe, medium setting) for fifteen one minute periods, with cooling as above.
The protein content of the product was determined (V D 6 (b)) and digests were prepared as in VI A 17(c). The hydrolysate was subjected to the Nelson test for reducing sugars (V D 3) and to the glucose oxidase/peroxidase test for free glucose (V D 4 (c)). The hydrolysate was subjected to paper chromatography in solvent (d), staining reagent (a) was employed.

(e) **Further modification of the attempted preparation of an exodextranase from B. megatherium**

An aliquot (1 ml) of the nutrient broth culture (VI A 17 (a)) was transferred aseptically to a cotton-wool-plugged 1l Erlemeyer flask containing medium M8 (250 ml). The flask was incubated at 30°C with orbital agitation. The experiment was stopped after 35 h. The bacterial cells were harvested by centrifugation at 2800 g. The cells were suspended in sterile water (8 ml) and inoculated into four cotton-wool-plugged 1l Erlemeyer flasks each containing medium M8 (250 ml).

One flask was used to monitor the growth of the Bacillus organism (V B 1 (d)) (figure VI 29). The flasks were incubated at 30°C with orbital agitation. The experiment was terminated after 32 h and the cells from the remaining three flasks were harvested by centrifugation at 2800 g. The resulting bacterial cells (10 g) were suspended in buffer B4 (25 ml) and disintegrated using the M.S.E. ultrasonic disintegrator (medium probe, setting medium) for twelve one minute periods with one minute periods of cooling in ice-water between each disintegration. The protein content of the resulting solution was determined ( V D 6 (b)). The resulting suspension was centrifuged at 2800 g.
Fig. VI 29  The growth curve of B. megatherium D₂ (VI A 17 (e))
The resulting solution was further centrifuged in a M.S.E. high speed centrifuge at 25,000 g for 2.5 h. Debris was obtained and the protein content of the clear supernatant was determined (V D 6 (b)).

Digests were prepared as in VI A 17 (c). The hydrolysate was subjected to the Nelson test for reducing sugars (V D 3) and to the glucose oxidase/peroxidase test for free glucose (V D 4 (c)). The product was also subjected to paper chromatography in solvent (d), using staining reagent (a).

VI A 18 Attempted preparation of a glucanhydrolase from swine kidney

(a) Initial preparation

Swine kidney (222 g) was mixed and homogenised at room temperature in buffer B6 (900 ml) and stirred at 2°C for 0.5 h. The product was centrifuged at 1700 g for 15 min and the pH of the supernatant was adjusted to pH 5.0 with glacial acetic acid (c. 2 ml). The resulting suspension was centrifuged at 1700 g for 20 min.

To the supernatant (1 l) ammonium sulphate A.R. (216 g) was added. The suspension was centrifuged at 2800 g at 0°C for 1.0 h. The precipitate was set aside.

To the supernatant ammonium sulphate A.R. (144 g) was added, and the resulting suspension was centrifuged at 2800 g. The resulting precipitate was dialysed against distilled water overnight.

The protein content of the crude enzyme preparation was determined (V D 6 (b)).
A digest was prepared as follows:

- buffer B5: 0.1 ml
- dextran T40: 16 mg
- crude enzyme preparation: 0.9 ml

The digest was incubated at 37°C for 24 h under a layer of toluene A.R. to discourage bacterial and mould growth.

An aliquot of the hydrolysate (0.1 ml) was subjected to the Nelson test for reducing sugars (V D 3) to detect enzyme activity.

Two further purifications of the crude enzyme preparation were performed. To the crude enzyme preparation ammonium sulphate A.R. (216 g/l) was added. The suspension was centrifuged. To the supernatant, ammonium sulphate A.R. (144 g/l) was added. The suspension was centrifuged. The centrifugate was dialysed against distilled water for 24 h.

This procedure was then repeated.

The final enzyme preparation was concentrated using an Amicon model 202 cell with a UM2 pad (cut-off $M_w = 1000$). The volume of solution was decreased from 250 to 20 ml.

The protein content of the enzyme preparation was determined (V D 6 (b)).

A digest was prepared as follows:

- buffer B5: 0.1 ml
- dextran T40: 15.5 mg
- enzyme preparation: 1.0 ml

The digest was incubated at 37°C under a layer of toluene A.R. to discourage bacterial and mould growth. Aliquots (0.1 ml) were
removed at intervals and subjected to the Nelson test for reducing sugars (VD 3). The results are shown in figure VI 30.

Samples of the enzymic hydrolysate were subjected to paper chromatography in solvent (a), (c) and (d). Staining reagent (a) was employed.

Further samples of the enzymic hydrolysate were subjected to thin layer chromatography (t.l.c.) in t.l.c. solvent (b). The results are shown in figure VI 31.

The product of a similar digest was subjected to gel permeation chromatography on a mixed bed Sephadex G-200/G-10 (1:1 w:w) column (VA 6 (c)). A Pharmacia K9/15 column was employed, the eluant was 1% sodium chloride solution and 0.6 ml (20 drop) fractions were collected. The results are shown in figure VI 32.

Digests were prepared as follows:-

buffer B4 0.05 ml
dextran T40 3.3 mg
enzyme preparation 0.2 ml

The pH of buffer B3 was increased from pH 3.4 to pH 7.0 in increments of 0.6 pH units for each of seven digests. The digests were incubated at 37°C for 24 h and the reducing sugar content of the hydrolysate was estimated using the Nelson reagents (VD 3). The pH optimum curve for the kidney dextranase is shown in figure VI 33.

(b) Further preparation of an exodextranase from swine kidney

The swine kidneys were kindly donated by Messrs Bowyers Ltd., London Road, Amersham.
Fig. VI 30 The attempted preparation of an exodextranase from swine kidney. Initial preparation. Release of reducing sugar from an enzyme digest.
Fig. VI 31 Preparation of an exodextranase from swine kidney.

t.l.c. of enzymic hydrolysate of Dextran T40

S substrate blank
E enzyme blank
G glucose standard
d total digest
t spots applied
f solvent front
Fig. VI 32 Attempted preparation of a glucanhydrolase from swine kidney.

Enzymic degradation of Dextran T 40. Gel permeation chromatography of the product on Sephadex G-200/G-10.

Dextran T40 ($\bar{M}_w = 40,000$) (substrate)

Dextran 10 ($\bar{M}_w = 10,000$)

6 hour enzymic hydrolysate

60 hour enzymic hydrolysate

fraction no.
Fig. VI.33 Attempted preparation of an exodextranase from swine kidney, pH optimum of the dextranhydrodase.
The enzyme was extracted as illustrated in the scheme shown in figure VI 34. All centrifugations were conducted at 2800 g.

Dialysis was performed against buffer B5 (5 l).

The protein contents of each product of the extraction procedure was determined. Digests were prepared as follows:

- buffer B5 0.1 ml
- dextran T40 15 mg
- extract 1.0 ml

The digests were incubated at 37°C for 15.5 h under a layer of toluene A.R. to discourage bacterial and mould growth. Aliquots (0.5 ml) were subjected to the Nelson test for reducing sugars (V D 3). The protein contents and the specific activities of each of the extracts are shown in table VI 22.

**Table VI 22** The protein contents and specific activities of the extracts from swine kidney

<table>
<thead>
<tr>
<th>Extract</th>
<th>Protein mg/ml</th>
<th>Specific activity (units/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Residue A</td>
<td>38.0</td>
<td>0</td>
</tr>
<tr>
<td>Centrifugate B</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Supernatant C</td>
<td>12.0</td>
<td>0</td>
</tr>
<tr>
<td>Centrifugate D</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Centrifugate E</td>
<td>51.0</td>
<td>0</td>
</tr>
<tr>
<td>Supernatant F</td>
<td>6.0</td>
<td>0</td>
</tr>
<tr>
<td>Centrifugate G</td>
<td>27.0</td>
<td>$5 \times 10^{-5}$</td>
</tr>
<tr>
<td>Centrifugate H</td>
<td>4.0</td>
<td>0</td>
</tr>
<tr>
<td>Supernatant I</td>
<td>6.0</td>
<td>$1.7 \times 10^{-5}$</td>
</tr>
<tr>
<td>Centrifugate J</td>
<td>3.0</td>
<td>$3 \times 10^{-5}$</td>
</tr>
<tr>
<td>Enzyme preparation</td>
<td>7.0</td>
<td>$1 \times 10^{-3}$</td>
</tr>
</tbody>
</table>
280

F ig u r e VI 34

The e x t r a c t i o n o f a g lu c a n h y d ro la s e from sw ine k id n e y
6 Swine k id n e y s (860 g)
b u f f e r B5 (860 g) a d d e d .
f o r 10 h .
C e n tr if u g e d

RESIDUE A

S to re d a t 0°C

SUPERNATANT
Ammonium s u lp h a te A .R . (216 g / l ) added
c e n t r if u g e d

CENTRIFUGATE

B
SUPERNATANT
Ammonium s u lp h a te A .R . ( l4 4 g / l ) ad d ed
c e n t r if u g e d
CENTRIFUGATE
d ia ly s e d
c e n t r if u g e d

CENTRIFUGATE

^

SUPERNATANT C

SUPERNATANT
Ammonium s u lp h a te A .R . ( 2 l6 g / l ) ad d ed
c e n t r if u g e d

CENTRIFUGATE

E

SUPERNATANT
Ammonium s u lp h a te A .R . ( l4 4 g / l ) added
c e n trifu g e d
CENTRIFUGATE

SUPERNATANT F

d ia ly s e d
c e n t r if u g e d
CENTRIFUGATE

G

SUPERNATANT
Ammonium s u lp h a te A .R. (2 1 6 g / l ) ad d ed
c e n t r if u g e d

CENTRIFUGATE

H

SUPERNATANT
Ammonium s u lp h a te A .R . ( l4 4 g / l ) added
c e n trifu g e d
CENTRIFUGATE
d ia ly s e d
c e n t r if u g e d

CENTRIFUGATE

J

Enzyme p r e p a r a t i o n

SUPERNATANT I


An aliquot of the enzyme solution (in buffer B5) (2 ml) was freeze-dried (VC 8). A further aliquot of the enzyme solution (2 ml) was used as a control.

Digests were prepared as follows:
freeze-dried enzyme and buffer B5 in distilled water (2 ml)  
[or enzyme in buffer B5 (2 ml)]

dextran T40  
30 mg

The digest was incubated at 37°C under a layer of toluene A.R. to discourage bacterial and mould growth.

Aliquots (0.1 ml) were subjected to the Nelson test for reducing sugars (VD 3) at intervals.

The results are shown in figure VI 35.

The enzymic hydrolysates were subjected to the qualitative test for glucose (VD 4 (c)) and to paper chromatography in solvents (a), (c) and (d) and to t.l.c. in t.l.c. solvent (b).

The product of a similar digest was subjected to gel permeation chromatography on a mixed bed Sephadex G-200/G-10 column. A Pharmacia K 19/15 column was employed, the eluant used was 1% sodium chloride solution and 0.6 ml (20 drop) fractions were collected.

The results after 75 h and 15 days are shown in figure VI 36.

Enzyme digests were prepared as follows:
carbohydrate  
20 mg
freeze-dried enzyme in buffer B5 (protein content 12 mg/ml)  
2 ml
sodium azide  
0.02%

The digests were incubated at 37°C. Aliquots (0.1 ml) were withdrawn at intervals and subjected to the Nelson test for reducing sugars (VD 3).
Fig. VI.35 The preparation of a glucanhydrolyase from swine kidney. Stability of the enzyme on freeze-drying.
Fig. VI 36 The preparation of an exodextranase from swine kidney. Molecular weight distribution of the product by gel permeation chromatography on Sephadex G-200/G-10.

dextran T40 (substrate)

glucose

75 hour digest

15 day digest
The results, shown as a percentage degradation, are shown in figure II 29 for GS-5B polysaccharide, *L. mesenteroides* NRRL B-512, *L. mesenteroides* NRRL B-1375 and acid-degraded *L. mesenteroides* NRRL B-512 dextrans.

**VI A 19** Acid hydrolysis at optimum conditions for the production of glucose from GS-5A polysaccharide

GS-5 A polysaccharide was hydrolysed by the method employed for GS-5 B polysaccharide (VI A 2). The results are tabulated in table VI 23.

**Table VI 23** Acid hydrolysis of the glucosidic linkages of GS-5A polysaccharide — Paper chromatography and electrophoresis of the product and reduced product

<table>
<thead>
<tr>
<th></th>
<th>solvent or buffer system</th>
<th>value</th>
<th>Intensity with staining reagent (a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$P_G$ product</td>
<td>solvent (d)</td>
<td>1.00</td>
<td>3+</td>
</tr>
<tr>
<td>$M_G$ product</td>
<td>buffer (a)</td>
<td>1.00</td>
<td>3+</td>
</tr>
<tr>
<td>$M_S$ reduced product</td>
<td>solvent (b)</td>
<td>0.99</td>
<td>4+</td>
</tr>
</tbody>
</table>

These results were obtained on the same chromatograms/electropherograms using the same amount of material, as the results tabulated in table VI 1 and hence comparison of the intensities, estimated by visual examination, is valid.
Acid hydrolysis at optimum conditions for the production of fructose from GS-5 A polysaccharide

The polysaccharide was hydrolysed by the method employed for GS-5 B polysaccharide (VI A3). The results are tabulated in table VI 24.

Table VI 24 Acid hydrolysed of the fructosidic linkages of GS-5 A polysaccharide - Paper chromatography and electrophoresis of the product and reduced product

<table>
<thead>
<tr>
<th></th>
<th>solvent or buffer system</th>
<th>value</th>
<th>Intensity</th>
<th>Staining reagent (a)</th>
<th>Staining reagent (e)</th>
</tr>
</thead>
<tbody>
<tr>
<td>E_g prod</td>
<td>solvent (a)</td>
<td>1.00</td>
<td>trace</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>solvent (d)</td>
<td>1.27</td>
<td>6+</td>
<td>4+</td>
<td></td>
</tr>
<tr>
<td>M_g prod</td>
<td>buffer (a)</td>
<td>1.00</td>
<td>trace</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>buffer (a)</td>
<td>0.91</td>
<td>6+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>M_s red</td>
<td>buffer (b)</td>
<td>1.00</td>
<td>5+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>prod</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M_g red</td>
<td>buffer (a)</td>
<td>0.83</td>
<td>2+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>prod</td>
<td></td>
<td>0.92</td>
<td>+</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

These results were obtained on the same chromatograms/electropherograms, using the same amount of material, as the results tabulated in table VI 2 and hence comparison of the intensities, estimated by visual inspection, is valid.

Composition of GS-5 A polysaccharide

The nitrogen, protein, carbohydrate, glucose, fructose and ash contents of GS-5 A polysaccharide (Batches 1 and 2) were determined by the methods employed for GS-5 B polysaccharide (VI A4). The results are tabulated in table II 15.
VI A 22 The methylation analysis of GS-5 A polysaccharide

GS-5 A polysaccharide (Batch 1) was methylated (VE 7) and a dry nujol mull of the methylated product was prepared. The mull was held between potassium bromide plates and the infrared spectrum in the region 3800-3000 cm\(^{-1}\) was obtained using a Perkin Elmer P.E. 325 infrared spectrophotometer (figure VI 37).

The remainder of the methylated product was hydrolysed (VE 8 (b)), reduced (VE 5) and acetylated (VE 9) and subjected to g.l.c. - m.s. analysis.

The g.l.c. trace illustrated (figure VI 38) was obtained on a Pye 104 double column gas chromatograph (VA 5(b) (i)) using packing material (ii). An oven temperature of 178° C was employed and a carrier gas flow rate of 44 cc/min was used.

G.l.c. data (not illustrated) were also obtained on the Perkin Elmer F11 g.l.c. coupled to the Hitachi RMS 4 m.s. using packing material (ii) (g.l.c. oven temperature 201° C). Components 2 and 3 were resolved by using an oven temperature of 170° C.

The mass spectra of components 1, 3, 4 and 5 are shown in figures VI 39 - VI 42. Component 2 was not an alditol acetate. The retention times and the molar proportions are shown in table II 16. The results obtained by the methylation analysis of a second batch of GS-5 A polysaccharide (Batch 2) are shown in table II 17. The chromatograms and spectra (essentially similar to those obtained from GS-5 A polysaccharide Batch 1) are not illustrated.
Fig. VII 37 The methylation of CS-5 A polysaccharide. The infrared spectrum of the methylated product.
Fig. VI 38 The g.l.c. trace of the acetylated, reduced, hydrolysed, methylated GS-5 A polysaccharide (Batch 1)
Fig. VI 39 The methylation analysis of GS-5 A polysaccharide (Batch 1). G.l.c.-m.s. Mass spectrum of component 1

% of base peak

m/e
Fig VI. 40 Mass spectrum of component 3
Fig. VII-42. Mass spectrum of component 5.
A qualitative *P. lilacinum* endodextranase digest of GS-5 A polysaccharide was prepared as for GS-5 B polysaccharide (VI A 14 (a)). The results of paper chromatography of the deionised enzymic hydrolysate are shown in table VI 25.

**Table VI 25** The *P. lilacinum* dextranase digest of GS-5 A polysaccharide - Paper chromatography of the enzymic hydrolysate

<table>
<thead>
<tr>
<th>Compound</th>
<th>$R_{1M_2}$</th>
<th>$R_{1M_1}$</th>
<th>Components of partial hydrolysate of Dextran T40</th>
<th>Intensity by visual inspection</th>
<th>Possible Identity</th>
</tr>
</thead>
<tbody>
<tr>
<td>31</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>glucose</td>
<td></td>
</tr>
<tr>
<td>32</td>
<td>0.98</td>
<td>1.00</td>
<td>+</td>
<td>isomaltose</td>
<td></td>
</tr>
<tr>
<td>33</td>
<td>0.58</td>
<td>0.58</td>
<td>+</td>
<td>isomaltotriose</td>
<td></td>
</tr>
<tr>
<td>34</td>
<td>0.236</td>
<td>0.21</td>
<td>+</td>
<td>'branch' pentasaccharide</td>
<td></td>
</tr>
<tr>
<td>35</td>
<td>0.15</td>
<td>0.13</td>
<td>+</td>
<td>'branch' hexasaccharide</td>
<td></td>
</tr>
<tr>
<td>36</td>
<td>0.099</td>
<td>0.082</td>
<td>+</td>
<td>'branch' heptasaccharide</td>
<td></td>
</tr>
<tr>
<td>37</td>
<td>0.063</td>
<td></td>
<td>+</td>
<td>'branch' octasaccharide</td>
<td></td>
</tr>
</tbody>
</table>

A quantitative *P. lilacinum* endodextranase digest of GS-5 A polysaccharide was prepared as described for GS-5 B polysaccharide (VI A 14 (b)) and the hydrolysate was analysed as described.

The results are shown in figure VI 43.
Fig. VI 43 The *P. lilacinium* dextranase digest of GS-5 A polysaccharide. Quantitative digest

- **standard dextran T40**
- **GS-5 A polysaccharide**
VI A 24 The swine kidney glucanhydrolase hydrolysis of GS-5 A polysaccharide

A quantitative swine kidney dextranase digest of GS-5 A polysaccharide was prepared as described for GS-5 B polysaccharide (VI A 18 (b)) and the hydrolysate was analysed as described.

The results are shown in figure II 35.

VI B The polysaccharide elaborated by S. mutans OMZ 51

VI B 1 The production, extraction and purification of the polysaccharide

The polysaccharide was cultured, extracted and purified as described (VE 2 and VE 3). The yields are shown in table III 1.

VI B 2 Acid hydrolysis of the optimum conditions for the production of glucose from OMZ 51 B polysaccharide

The polysaccharide was hydrolysed as described (VE 4 (a)) and the neutralised hydrolysate was subjected to paper chromatography in solvent (d). Staining reagent (a) was employed. The results are shown in table VI 26.

Table VI 26 Acid hydrolysis of the glucosidic linkages of OMZ 51 B polysaccharide - paper chromatography

<table>
<thead>
<tr>
<th>solvent system</th>
<th>$R_g$ value</th>
<th>intensity</th>
<th>possible identity</th>
</tr>
</thead>
<tbody>
<tr>
<td>(d)</td>
<td>1.01</td>
<td>3+</td>
<td>glucose</td>
</tr>
</tbody>
</table>
VI B 3 Acid hydrolysis at the optimum conditions for the production of fructose from OMZ 51B polysaccharide

The polysaccharide was hydrolysed as described (V E 4 (b)) and the product was subjected to paper chromatography in solvent (d). Staining reagent (a) was employed. The results are shown in table VI 27.

Table VI 27 Acid hydrolysis of the fructosidic linkages of OMZ 51B polysaccharide - Paper chromatography

<table>
<thead>
<tr>
<th>solvent system</th>
<th>$R_G$ value</th>
<th>intensity</th>
<th>possible identity</th>
</tr>
</thead>
<tbody>
<tr>
<td>(d)</td>
<td>1.25</td>
<td>3+</td>
<td>fructose</td>
</tr>
<tr>
<td>(d)</td>
<td>1.00</td>
<td>+</td>
<td>glucose</td>
</tr>
</tbody>
</table>

VI B 4 Composition of OMZ 51B polysaccharide

The nitrogen, protein, carbohydrate, glucose, fructose and ash contents of OMZ 51B polysaccharide were determined by the methods employed for GS-5 B polysaccharide (V A 4). The results are shown in table III 2.

VI B 5 The methylation analysis of OMZ 51B polysaccharide

OMZ 51B polysaccharide was methylated, treated and analysed as in VI A 22 and the g.l.c. trace is shown in figure VI 44. The mass spectra are shown in figures VI 45 - VI 47. The retention times and the molar proportions of each component are shown in table III 3. The infrared spectrum of the methylated polysaccharide was produced as described for GS-5 A polysaccharide (VI A 22). The spectrum is shown in figure VI 48.
Fig. VI.45 The methylolation analysis of CMZ 51 B polysaccharide. G.L.C.-m.s. Mass spectrum of component 1.
Fig. VI 47  Mass spectrum of component 4
Fig. VI 48. The methylation of CM2 51 B polysaccharide. The infrared spectrum of the methylated product.
VI B 6 The P. lilacinum dextranase digest of OMZ 51B polysaccharide

A qualitative P. lilacinum endodextranase digest of OMZ 51B polysaccharide was prepared and analysed as described for GS-5B polysaccharide (VI A 14 (a)). The results of paper chromatography of the deionised enzyme hydrolysate are shown in table VI 28.

Table VI 28 The P. lilacinum dextranase digest of OMZ 51B polysaccharide - Paper chromatography of the enzymic hydrolysate

<table>
<thead>
<tr>
<th>Compound</th>
<th>$R_1^{M_2}$</th>
<th>$R_1^{M_2}$</th>
<th>components of partial hydrolysate inspection</th>
<th>Intensity by visual inspection of Dextran T40</th>
<th>Possible identity</th>
</tr>
</thead>
<tbody>
<tr>
<td>41</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>glucose</td>
<td></td>
</tr>
<tr>
<td>42</td>
<td>0.96</td>
<td>1.00</td>
<td>++</td>
<td>isomaltose</td>
<td></td>
</tr>
<tr>
<td>43</td>
<td>0.49</td>
<td>0.49</td>
<td>++</td>
<td>isomaltotriose</td>
<td></td>
</tr>
<tr>
<td>44</td>
<td>0.25</td>
<td>-</td>
<td></td>
<td>isomaltotetraose</td>
<td></td>
</tr>
<tr>
<td>45</td>
<td>0.16</td>
<td>-</td>
<td>+</td>
<td>'branch' penta-saccharide</td>
<td></td>
</tr>
<tr>
<td>46</td>
<td>-</td>
<td>0.14</td>
<td></td>
<td>isomaltopentaose</td>
<td></td>
</tr>
<tr>
<td>47</td>
<td>0.09</td>
<td>-</td>
<td>+</td>
<td>'branch' hexa-saccharide</td>
<td></td>
</tr>
<tr>
<td>48</td>
<td>-</td>
<td>0.068</td>
<td></td>
<td>isomaltohexaose</td>
<td></td>
</tr>
<tr>
<td>49</td>
<td>0.05</td>
<td>-</td>
<td>+</td>
<td>'branch' hepta-saccharide</td>
<td></td>
</tr>
</tbody>
</table>

A quantitative P. lilacinum endodextranase digest of OMZ 51B polysaccharide was prepared and analysed as described for GS-5B polysaccharide (VI A 14 (b)). The results are shown in figure VI 49.
Fig. VI 49  The *P. lilacinum* dextranase digest of OMZ 51 B polysaccharide. Quantitative digest

Reducing sugar as μg glucose per 100μl aliquot of solution

Time (hours)
VI B 7. The swine kidney glucanhydrolase hydrolysis of OMZ 51B polysaccharide

A quantitative swine kidney glucanhydrolase digest of OMZ 51B polysaccharide was prepared and analysed as described for GS-5 B polysaccharide (VI A 18 (b)).

The results are shown in figure III 3.

VI B 8. The gel permeation chromatography of OMZ 51B polysaccharide on Sephadex G-200

OMZ 51B polysaccharide was subjected to gel permeation chromatography on Sephadex G-200 using a Pharmacia K 26/100 column (V A 6). The carbohydrate content of each fraction was determined (V D 2) and absorbances were determined on an Eel spectrophotometer. The elution diagram is shown in figure III 4.

VI B 9. Acid hydrolysis at the optimum conditions for the production of glucose from OMZ 51A polysaccharide

The polysaccharide was hydrolysed as described for GS-5 B polysaccharide (V E 4(a)) and the neutralised hydrolysate was subjected to paper chromatography in solvent (d). Staining reagent (a) was employed.

The results are shown in table VI 29.

Table VI 29 Acid hydrolysis of the glucosidic linkages of OMZ 51A polysaccharide – Paper chromatography

<table>
<thead>
<tr>
<th>Solvent system</th>
<th>R&lt;sub&gt;g&lt;/sub&gt; value</th>
<th>intensity</th>
<th>possible identity</th>
</tr>
</thead>
<tbody>
<tr>
<td>(d)</td>
<td>0.99</td>
<td>3+</td>
<td>glucose</td>
</tr>
</tbody>
</table>
VI B 10  Acid hydrolysis at the optimum conditions for the production of glucose from OMZ 51A polysaccharide

The polysaccharide was hydrolysed as described for GS-5B polysaccharide (V E 4(b)) and the neutralised hydrolysate was subjected to paper chromatography in solvent (d). Staining reagent (a) was employed. The results are shown in table VI 30.

Table VI 30. Acid hydrolysis of the fructosidic linkages of OMZ 51A polysaccharide—Paper chromatography

<table>
<thead>
<tr>
<th>Solvent system</th>
<th>E_g value</th>
<th>intensity</th>
<th>possible identity</th>
</tr>
</thead>
<tbody>
<tr>
<td>(d)</td>
<td>1.25</td>
<td>trace</td>
<td>fructose</td>
</tr>
<tr>
<td>(d)</td>
<td>1.00</td>
<td>+</td>
<td>glucose</td>
</tr>
</tbody>
</table>

The results shown in tables VI 29 and VI 30 were obtained on the same chromatograms as those shown in tables VI 26 and VI 27 and hence comparison of the intensities, estimated by visual examination, is valid.

VI B 11 Composition of OMZ 51A polysaccharide

The nitrogen, protein, carbohydrate, glucose, fructose and ash contents of OMZ 51A polysaccharide were determined by the methods as employed for GS-5 B polysaccharide (VI A 4). The results are shown in table III 4.

VI B 12 The methylation analysis of OMZ 51A polysaccharide

OMZ 51A polysaccharide was methylated and analysed as for GS-5A polysaccharide (VI A 22). The g.l.c. trace is shown in figure VI 50. The mass spectra are shown in figures VI 51 to VI 54.
The retention times and the molar proportions of each component are shown in table III 5. The infrared spectrum of the methylated polysaccharide was prepared as described for GS-5A polysaccharide (VI A 22). The infrared spectrum is shown in figure VI 55.

VI B 13 The P. lilacinum dextranase digest of OMZ 51A polysaccharide

A qualitative P. lilacinum endodextranase digest of OMZ 51A polysaccharide was prepared as described for GS-5B polysaccharide (VI A 14 (a)). The result of paper chromatography of the deionised enzymic hydrolysate are shown in table VI 31.

Table VI 31. The P. lilacinum dextranase digest of OMZ 51A polysaccharide—Paper chromatography of the enzymic hydrolysate

<table>
<thead>
<tr>
<th>Compound</th>
<th>R'M_2</th>
<th>R'M_2 components of partial hydrolysate of Dextran T40</th>
<th>Intensity by visual inspection of inspection</th>
<th>Possible identity</th>
</tr>
</thead>
<tbody>
<tr>
<td>51</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>52</td>
<td>0.96</td>
<td>1.00</td>
<td>++</td>
<td>glucose</td>
</tr>
<tr>
<td>53</td>
<td>0.49</td>
<td>0.49</td>
<td>++</td>
<td>isomaltose</td>
</tr>
<tr>
<td>54</td>
<td>0.28</td>
<td>trace</td>
<td>trace</td>
<td>isomaltotriose</td>
</tr>
<tr>
<td>55</td>
<td></td>
<td>0.25</td>
<td>isomaltotetraose</td>
<td>'branch' tetra-saccharide</td>
</tr>
<tr>
<td>56</td>
<td>0.16</td>
<td>+</td>
<td>+</td>
<td>isomaltpentaose</td>
</tr>
<tr>
<td>57</td>
<td></td>
<td>0.14</td>
<td>'branch' penta-saccharide</td>
<td></td>
</tr>
<tr>
<td>58</td>
<td>0.09</td>
<td>+</td>
<td>'branch' hexa-saccharide</td>
<td></td>
</tr>
<tr>
<td>59</td>
<td></td>
<td>0.068</td>
<td>isomaltohexaose</td>
<td></td>
</tr>
<tr>
<td>0.05</td>
<td></td>
<td>+</td>
<td>'branch' hepta-saccharide</td>
<td></td>
</tr>
</tbody>
</table>
Fig. VI 50  The g.l.c. trace of the acetylated, reduced, hydrolysed, methylated OMZ 51 A polysaccharide
Fig. VI 51 The methylation analysis of OMZ 51 A polysaccharide. G.I.c.-m.s. Mass spectrum of component 1
Fig. VI. 52. Mass spectrum of component 3.
Fig. VI 53 Mass spectrum of component 4.

[Graph showing mass spectrum with peaks at m/e values of 43, 101, 117, 129, 161, and 189.]
Fig. VI. 54  Mass spectrum of component 5
Fig. VI 55 The methylation of OMZ 51 A polysaccharide. The infrared spectrum of the methylated product.

Wave number cm⁻¹

Native OMZ 51 A polysaccharide

Methylated polysaccharide
Fig. VI 56  The *P. lilacinium* dextranase digest of OMZ51 A polysaccharide. Quantitative digest

Reducing sugar as 
µg glucose per 100µl aliquot of solution

Standard Dextran T40

OMZ 51 A polysaccharide

Time (hours) 50
A quantitative *P. lilacinum* endodextranase digest of OMZ 51A polysaccharide was prepared and analysed as described for GS-5 B polysaccharide (VI A 14 (b)).

The results are shown in figure VI 56.

**VI B 14** The swine kidney glucanhydrolase hydrolysis of OMZ 51A polysaccharide

A quantitative swine kidney glucanhydrolase digest of OMZ 51A polysaccharide was prepared and analysed as for GS-5 B polysaccharide (VI A 18 (b)).

The results are shown in figure III 2.

**VI C** The swine kidney glucanhydrolase hydrolysis of several bacterial polysaccharides and other carbohydrates

The swine kidney glucanhydrolase was prepared as described (VI A 18 (b)). Digests were prepared as follows:

<table>
<thead>
<tr>
<th>Carbohydrate</th>
<th>20 mg (weight accurately known)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Swine kidney glucanhydrolase</td>
<td>2 ml</td>
</tr>
<tr>
<td>in buffer B5</td>
<td>(protein concentration 12 mg/ml)</td>
</tr>
<tr>
<td>Sodium azide</td>
<td>0.02%</td>
</tr>
</tbody>
</table>

The digests were incubated at 37°C for 500 h.

Aliquots (0.1 ml) of the digest were taken at intervals and subjected to the Nelson test for reducing sugars (V D 3).

The extent of enzymic hydrolysis (conversion to glucose) was calculated from the weight of starting material (corrected for carbohydrate content) and the amount of reducing sugar released (estimated as glucose).
The results are shown in figures IV 1 - IV 3. After 500 hours, the digests were subjected to thin layer chromatography in solvent (b). The chromatograms are illustrated in figure IV 4.

Most of the carbohydrates investigated were obtained from the departmental collection. Potato amylose was kindly donated by Mr. P.A. Leonard, S. salivarius fructan by Mr. K. Marshall. Sucrose A.R. was obtained from B.D.H. Ltd., and Dextran T40 from Sigma Biochemicals Ltd.
CHAPTER VII

APPENDICES

Appendix A  Modification of the Perkin Elmer - Hitachi g.l.c. - m.s. Interface

The original Perkin Elmer - Hitachi g.l.c. - m.s. interface is illustrated in figure VII 2 and it functioned as follows. A sample was injected onto a 6 ft stainless steel, narrow-bore column (packing material (i)). On leaving the g.l.c. column the eluant gas was split, part going to the g.l.c. flame ionisation detector (f.i.d.) and part going into the m.s. The ratio of the two fractions, governed by the size of the restrictor adjacent to the separator, was 1:10 g.l.c.: m.s. That portion of the eluant gas going to the m.s. passed into the m.s. \( \text{via} \) a 1M stainless steel, 0.01" internal diameter, heated line and a Watson-Bieman separator, where the majority of the carrier gas was pumped away. The sample on entering the evacuated m.s. chamber was ionised by electron impact and the ions produced were monitored, as they passed down the flight tube, on a total ion monitor (t.i.m.) (figure VII 4). The spectra were displayed on a S.E. 3006 U.V. recorder.

\( 1,5-\text{Di-O-acetyl-2,3,4,6-tetra-O-methyl-\( \beta \)-glucitol (TMG) } \) was injected onto the column. Both the g.l.c. and the t.i.m. traces were broad and featureless. It was postulated that chemical degradation was occurring on the stainless steel of the column and replacement of the stainless steel column by a glass column (involving replacement of the analyser head) produced an immediate improvement in the g.l.c. trace but the t.i.m. trace remained broad.

Modifications of the interface itself were then initiated (figures VII 1 and VII 3). Wherever possible stainless steel was replaced by
glass. The heated line was removed and the separator was placed flush with the g.l.c. oven. A fully-insulated extension unit was built into the g.l.c. oven, so increasing the oven volume, and allowing room for a coupling to be inserted. The Perkin Elmer high performance oven was replaced by a Perkin Elmer precision oven, which is fitted with a rear door that permits easy access. With the oven extension in place no temperature drop across the modified g.l.c. oven could be detected. A metal coupling was fitted to the glass line by a glass-to-metal seal and a glass restrictor, consisting of a fine capillary, was blown into the line. The restrictor, prepared in our workshop, was produced to give a flow rate slightly greater than that required on the machine (10 ml/min). It was blown into the glass line and the final flow rate was adjusted in situ by heating the restrictor gently with a cool gas-oxygen flame, so collapsing the capillary slightly. In addition to reducing degradation on the stainless steel surface, the glass restrictor can often be unblocked with a 'tezler', should it become constricted.

These modifications completed, the apparatus gave good results for a period of several weeks of constant use. However, a gradual deterioration in the standard of the g.l.c. traces was noted and the t.i.m. traces were also slightly broadened. Cleaning of the splitter and the f.i.d. jet revealed that some build up of material, thought to have originated from leakage of stationary phase (i) from the g.l.c. column, had occurred. Other workers\textsuperscript{421} had also encountered problems with leakage of this stationary phase and had overcome the problem by substituting stationary phase (ii). After this substitution had been made in our own apparatus and after clearing the f.i.d. jet and the splitter, the apparatus functioned satisfactorily for a further two months.
After this time some broadening, not as severe as previously, of the g.l.c. trace was noted. The t.i.m. was unaffected. This broadening resulted from material being deposited in the f.i.d. jet due to an inadequate carrier gas pressure. Since the pressure of the dry helium carrier gas could not be increased (this would alter the amount of material going to the m.s.) it was decided to increase that of the hydrogen. Thus, the hydrogen gas acts as an additional carrier gas in the final stages of the g.l.c. system. This completely overcame the problem of deposition of material at the f.i.d. jet but had the effect of decreasing the relative retention time of any component with an absolute retention time greater than that of t.m.g., and although used in the earlier experiments the retention times of the components were not totally reproducible. In the later part of the work the retention times of components were separately determined on a Pye 104 gas chromatograph.
Legend to Figure VII 1

1 Hitachi dual pen recorder
2 Perkin Elmer gas regulator
3 Perkin Elmer gas chromatograph analyser head for glass columns (part no. 0454-0207)
4 Gas chromatograph oven extension unit
5 Perkin Elmer precision gas chromatograph oven (part no. 0454-0001)
6 Watson-Bieman separator
7 Hitachi RMS 4 mass spectrometer
8 Bradley Electronics D.V.M. 173 digital voltmeter
9 S.E. 3006 ultra-violet recorder
10 Perkin Elmer ion monitor and heater console
Fig. VII 2 The original Perkin Elmer-Hitachi g.l.c.-m.s. interface

- Injection port
- Flame ionisation detector
- Heated line (1M)
- Splitter (stainless steel)
- Column (stainless steel)
- Insulation
- Air circulation system
- Perkin Elmer F11 g.l.c. system
- 0.01" int. diam. stainless steel expansion coil
- 0.01" int. diam. stainless steel crimped tube restrictor
- Pump
- Watson-Biemann separator
Fig. VII 3 The modified Perkin Elmer-Hitachi g.l.c.-mass spectrometer

- Injection port
- Flame ionisation detector
- Glass-metal seal
- Restrictor
- To pump
- Splitter (stainless steel)
- Coupling (brass)
- Column (glass, narrow bore)
- Rear door
- Air circulation system

G.L.C. system Perkin Elmer F11
Watson-Rieman separator
Fig. VII 4 Ion trajectory, Hitachi R.M.S. 4 mass spectrometer

- to pump
- main magnet
- magnetic field
- analyser (flight tube)
- slits
- total ion monitor
- solid injection port
- multiplier
- ion source
Appendix B  Comparison of the proportions of two components in a synthetic mixture as determined by g.l.c. and classical methods

In the early methylation experiments the g.l.c. traces of the modified Perkin Elmer - Hitachi g.l.c. - m.s. system were used to determine both relative retention times and molar proportions of the components of methylation analysis. The g.l.c. - m.s. interface having been substantially modified, it was essential to ensure that the proportions of the various components being obtained were the same as those in the original mixtures being analysed. In order to investigate this, a synthetic mixture of a di-O-methyl-D-glucose and a tetra-O-methyl-D-glucose was prepared and the molar proportions of the two components were determined by g.l.c. and classical methods.

VII B 1 Preparation of a synthetic mixture of 2,3,4,6-tetra-O-methyl-D-glucose and 3,4-di-O-methyl-D-glucose

The mixture was prepared by mixing c. 20 mg of each component and dissolving the whole in water (10 ml).

VII B 2 Estimation of the synthetic mixture by hypoiodite oxidation

(a) Preparation of a standard graph

Solutions were prepared containing 0.25, 0.5, 1.0, 1.5, 2.0 and 2.5 mg of 2,3,4,6-tetra-O-methyl-D-glucose in water (5 ml).

A 0.1M solution of iodine and a 0.1M solution of sodium thiosulphate were prepared from standard ampoules and a solution 0.2M with respect to both sodium bicarbonate and sodium carbonate (pH 10.6) was prepared.

0.1M Iodine (1 ml) was pipetted into the sugar solutions (5 ml) contained in B24 stoppered pyrex tubes 0.2M sodium bicarbonate-sodium
carbonate solution (2 ml) was added, the stoppers were moistened with a little 70% potassium iodide solution, and the reactions were allowed to proceed, in the dark, for 2-2.5 h, at room temperature. The stoppers were then washed with distilled water and the washings were placed in the reaction vessel.

The solutions were acidified with $\frac{M}{2}$ sulphuric acid (1 ml) and diluted to 20 ml. They were mixed by inversion and titrated against 0.01$M$ sodium thiosulphate using 1% sodium starch glycollate (1 ml) as indicator.

The standard graph is shown in figure VII 5.

(b) Estimation of the synthetic mixture

Half of the solution of the standard mixture was spotted onto a washed Whatmans No.3 paper and developed in solvent (c). Test strips were cut and were stained in reagent (d). The two components were then eluted from the paper and estimated as in VII B 2(a). The results are shown in table VII 1.

<table>
<thead>
<tr>
<th>Table VII 1</th>
<th>Estimation of a synthetic mixture by hypoiodite oxidation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sodium thio-thio consumed (ml)</td>
</tr>
<tr>
<td>Blank</td>
<td>10.4</td>
</tr>
<tr>
<td>2,3,4,6-tetra-O-methyl-$D$-glucose</td>
<td>9.15</td>
</tr>
<tr>
<td>3,4-di-O-methyl-$D$-glucose</td>
<td>8.78</td>
</tr>
</tbody>
</table>

The molar ratio of 2,3,4,6-tetra-O-methyl-$D$-glucose : 3,4-di-O-methyl-$D$-glucose = 100:129.
VII B 3 Estimation of the synthetic mixture by g.l.c.

The synthetic mixture was reduced and acetylated as in V E 5 and V E 9 respectively, and subjected to g.l.c. on the modified Perkin Elmer - Hitachi g.l.c.-m.s. apparatus.

The chromatogram is shown in figure VII 6 and the areas under the peaks are given in table VII 2.

Table VII 2 Estimation of a synthetic mixture by g.l.c.

<table>
<thead>
<tr>
<th></th>
<th>Area under the peak</th>
<th>Molar proportion</th>
</tr>
</thead>
<tbody>
<tr>
<td>1,5 di-O-acetyl-2,3,4,6-tetra-O-methyl-D-glucitol</td>
<td>135</td>
<td>100</td>
</tr>
<tr>
<td>1,2,5,6-tetra-O-acetyl-3,4-di-O-methyl-D-glucitol</td>
<td>194</td>
<td>121</td>
</tr>
</tbody>
</table>

The molar ratio of 1,5-di-O-acetyl-2,3,4,6-tetra-O-methyl-D-glucitol : 1,2,5,6-tetra-O-acetyl-3,4-di-O-methyl-D-glucitol = 100:122.

Comparison of the proportions of the two components in the synthetic mixture as determined by the two methods shows close agreement.

Thus it appears that modification of the g.l.c.-m.s. interface did not alter the response of the apparatus to different components.
Fig. VII 5 Standard graph for hypiodite reagents
Fig. VII 6  g.l.c. trace of acetylated, reduced synthetic mixture

1,5-di-O-acetyl-2,3,4,6-tetra-O-methyl-D-glucitol

1,2,5,6-tetra-O-acetyl-3,4-di-O-methyl-D-glucitol

Absolute Retention Time (minutes)
Appendix C  The Mass Spectra of Some Standard Glucitol Derivatives as Determined on the Modified Perkin Elmer - Hitachi g.l.c. - m.s.
Fig. VII 7 The mass spectrum of 1,5-di-O-acetyl-2,3,4,6-tetra-O-methyl-D-glucitol

\[
\begin{align*}
\text{CH}_2\text{OAc} & \\
\text{MeO} & 117 \\
\text{OMe} & 205 \\
\text{OAc} & 161 \\
\text{CH}_2\text{OME} & 45 \\
\end{align*}
\]

% of base peak

\[
\begin{align*}
100 & \\
43 & \\
45 & \\
101 & \\
117 & \\
129 & \\
145 & \\
161 & \\
205 & \\
\end{align*}
\]

\[
\begin{align*}
205 & \rightarrow 145 \\
161 & \rightarrow 129 \\
161 & \rightarrow 101 \\
117 & \rightarrow 87 \\
101 & \rightarrow 71 \\
\end{align*}
\]
Fig. VII 8 The mass spectrum of 1,2,5-tri-O-acetyl-3,4,6-tri-O-methyl-D-glucose

CH₂OAc

MeO

CH₂OMe

189  →  129
161  →  101
129  →  99
129  →  87
Fig. VII 9  The mass spectrum of 1,3,5-tri-O-acetyl-2,4,6-tri-O-methyl-D-glucitol

CH₂OAc

AcO

CH₂OMe

161  129
161  101
129  87
117  87
101  71
233

45

101
87
71
Fig. VII 10  The mass spectrum of 1,4,5-tri-O-acetyl-2,3,6-tri-O-methyl-D-glucitol

CH₂OAc

-OMe  117

MeO

-OAc

-OAc

CH₂OMe  45

233  -(60+60)  113
233  -(60+30*42)  101
233  -(42+32)  99
117  -30  87

m/e
Fig. VII 11  The mass spectrum of 1,5,6-tri-O-acetyl-2,3,4-tri-O-methyl-D-glucitol

\[
\begin{align*}
&\text{CH}_2\text{OAc} \\
&\text{OMe} \quad 117 \\
&\text{MeO} \quad 161 \quad 233 \\
&\text{OMe} \quad 189 \\
&\text{OAc} \\
&\text{CH}_2\text{OAc} \\
&189 \quad -60 \rightarrow 129 \\
&161 \quad -60 \rightarrow 101 \\
&129 \quad -30 \rightarrow 99 \\
&129 \quad -42 \rightarrow 87 \\
&117 \quad -30 \rightarrow 87
\end{align*}
\]
Fig. VII 12 The mass spectrum of 1,2,5,6-tetra-O-acetyl-3,4-di-O-methyl-D-glucitol

\[ \text{CH}_2\text{OAc} \]
\[ \text{OAc} \]
\[ \text{MeO} \]
\[ \text{OMe} \]
\[ \text{OAc} \]
\[ \text{CH}_2\text{OAc} \]

189 $\rightarrow$ 129
189 $\rightarrow$ 99
189 $\rightarrow$ 87
Fig. VII 13. The mass spectrum of 1,3,5,6-tetra-o-acetyl-2,4-di-O-methyl-D-glucitol.
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