The polysaccharides from the envelopes of heterocysts and spores of *Anabaena cylindrica* consist of repeating units containing 1 mannosyl and 3 glucosyl residues, all linked by β(1 → 3) glycosidic bonds, with glucose, xylose, galactose, and mannose present in side branches. Degradation of the polysaccharides with specific glycosidases has permitted identification of the linkages to almost all of the branches.

When the polysaccharides, from which all but two types of side branches had been cleaved, were digested with a β(1 → 3) endoglucanase, glucose, a tri, and a pentasaccharide were produced. The oligosaccharide products were identified as

\[
\begin{align*}
\text{Glc} & \quad \text{Xyl} \\
\downarrow & \quad \downarrow \\
1 & \quad 4 & 1 & \quad 4 \\
\text{Man} & \quad \text{Glc} & \quad \text{Glc} & \quad \text{Glc}
\end{align*}
\]

The backbone of the polysaccharides were sequenced from the reducing terminus by a modified Smith degradation. Analysis with NaB\(_3\)H\(_4\) at each stage of the degradation showed that the backbones terminate in the sequence Man-Glc-Glc-Glc and are therefore presumed to have the structure (Man-Glc-Glc-Glc), and that they contain an average of from 128 to 150 sugar residues. From the information obtained, the repeating sequences of the original polysaccharides from the two types of differentiated cells of *A. cylindrica* could be largely deduced and appeared to be identical.

The polysaccharides from the envelopes of differentiated cells, heterocysts and spores (akinetes), of the blue-green alga (cyanobacterium) *Anabaena cylindrica* have identical linkages, as determined by methylation analyses. The sugars in their backbones, linked by β(1 → 3) glycosidic bonds, are present as repetitions of a tetrasaccharide containing 1 mannosyl and 3 glucosyl residues. One of every 3 glucosyl residues in the backbones is branched at both positions 2 and 4. Approximately half of the remaining glucosyl residues in the backbones have a side branch (1).

We have continued the analysis of these polysaccharides with the goals of determining whether, as the earlier results had suggested, they are essentially identical and of identifying a repeating subunit which would presumably be synthesized or activated during differentiation in *A. cylindrica*.

**MATERIALS AND METHODS**

**Preparation of Polysaccharides**

Lipid-free envelope layers of heterocysts and spores of *A. cylindrica* Lemm. (A.T.C.C. 29414) were prepared as described earlier (1). The polysaccharides were extracted by boiling 50 mg of lipid-free envelopes in 50 ml of 5% Na\(_2\)PO\(_4\) for 6 min (4). The suspension was centrifuged at 10,000 × g for 5 min, and the pellet was washed by centrifuging as before for 2 to 3 times with 5 ml of distilled water. The combined supernatant fluids were dialyzed for 24 h against three changes of distilled water, concentrated to 5 ml in a rotary evaporator, and lyophilized. The lyophilized material was maintained under vacuum at 60°C.

**Enzymatic Hydrolysis of the Branches of the Polysaccharide**

Lyophilized polysaccharide, different glycosidases, and buffers (see below) were incubated in the ratio 0.5 mg:1.0 mg:1 ml, at the appropriate temperature, with continuous agitation. After 0, 1, 3, 6, 10, and 24 h of incubation, 10-μl aliquots of the reaction mixture were diluted with 0.99 ml of distilled water, and tested for the release of reducing groups by Nelson's test (5). At the end of 24 h, the reaction mixture was boiled, centrifuged at 10,000 × g for 5 min, and the pellet was washed by centrifuging as before for 2 to 3 times with 10 ml of distilled water. The supernatant fluids were divided, reduced in volume to 1 ml in a rotary evaporator, and their sugar composition was analyzed by gas-liquid chromatography (1, 6). The pellet was divided into three portions; two portions of 4 mg each were used for sugar composition analysis and for methylation analysis (1, 7), and the major, third portion was subjected to the action of other glycosidases. The glycosidases and reaction conditions used which resulted in partial degradation of the polysaccharides were:

1. β-Glucosidase from almonds, obtained and used as described previously (1).
2. α-Galactosidase (obtained from Boehringer Mannheim GmbH, Germany) from green coffee beans, used in a 2:1 v/v mixture of 1.3 g of K\(_2\)HPO\(_4\)/100 ml and 2.28 g of KH\(_2\)PO\(_4\)/100 ml, pH 6.5, at 37°C. The enzyme was shown to be active on β-nitrophenyl-α-D-galactoside (obtained from Sigma Chemical Co., St. Louis, MO) and inactive on lactose.
3. β-Mannosidase-containing β-mannanase S339Q from *Penicillium ochro-chloron*, obtained from Dr. E. T. Reese (United States Army Natick Laboratories, Natick, MA), and used as described previously (1).
4. α-Glucosidase from yeast, obtained and used as described previously (1).
5. β-Xylosidase (from Sigma Chemical Co.) from *Aspergillus niger*, used in 0.5 M sodium acetate, pH 5.2, at 25°C. The enzyme was shown to be active on β-phenylxyloside (also obtained from Sigma,

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Liliana Cardemil  
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C. Peter Wolk‡  
*From the MSU-DOE Plant Research Laboratory, Michigan State University, East Lansing, Michigan 48824*
Chemical Co.) and to be inactive on α(1 → 6)glucosyl xylitol (a generous gift from Dr. P. Koosman, Technische Hogeschool Delft, The Netherlands (8)).

Other enzymes tested which did not degrade the polysaccharides were:

6. β-Galactosidase (obtained from Sigma Chemical Co.) from Escherichia coli, used in a 2:1 v/v mixture of 0.25 M NaH₂PO₄ and 0.25 M Na₂HPO₄, adjusted to pH 7.5, at 37°C. The enzyme was shown to be active on lactose and to be inactive on β-nitrophenyl-β-D-galactoside.

7. 8. β-Mannosidase from Polyporus sulphureus (9) and α-mannosidase from jack beans (10), obtained from Dr. Yu-Teh Li (Tulane University, New Orleans, LA), and used as described previously (1).

**Determination of the Repeating Sequences of the Original Polysaccharides by Cleavage, with 8(1 → 3)Endoglucomannan, of the Partially Degraded Polysaccharides**

After partial degradation of the polysaccharides from heterocysts and spores by β-glucosidase, α-galactosidase, β-mannosidase-containing β-mannanase, and α-glucosidase, 12 mg of the residual polysaccharide were digested by 6 mg of β(1 → 3)endoglucanase S₁₀₀₀N in 0.01 M sodium acetate, pH 5, at 50°C. The enzyme, partially purified from Rhizopus arrhizus QM-1032 (11), was obtained from Dr. Reese. The time course of the reaction was followed by Nelson's test (5), and the reaction was stopped by boiling after either 48 h (on the first occasion) or 20 h (thereafter). As a control, laminarin was incubated with the enzyme under the same conditions. Each reaction mixture, concentrated to 2 ml in a rotary evaporator, was passed through a Bio-Gel P-2 column as described previously (1). Fractions of 1.5 ml were collected and assayed for the presence of sugars by the anthrone test (12).

Three peaks were found: a monosaccharide fraction, a trisaccharide fraction, and an oligosaccharide fraction of higher molecular weight.

The monosaccharide fraction was analyzed only for sugar composition. Of the 4 mg of material in each of the two oligosaccharide fractions, 0.5 mg was used for sugar analysis, 3.0 mg were used for methylation analysis, and 0.5 mg was subjected to electrophoresis on paper in 0.05 M sodium acetate, pH 9.2, at 20 V/cm (1) for 18 h. The one oligosaccharide found in each fraction was eluted from the paper and subjected to enzymatic hydrolysis with α- and β-glucosidase and with β-xylanase, as described above.

**Reducing-end Analyses**

**Reduction**—Sixty milligrams of lipid-free envelopes from heterocysts and spores were subjected to Smith degradation (13) with sodium periodate in darkness at 5°C for 6 days, as described previously (1). The backbone polysaccharide purified from the reaction mixture was lyophilized and weighed. Four milligrams of this white material were reduced with 5 mg of NaBH₄ (186 mCi/mmol, New England Nuclear, Boston, MA) in 1 ml of 1 M NaOH for 1 h at room temperature. The reaction was stopped with glacial acetic acid, and the solution was dried under air in a water bath at 60°C. The polysaccharide was then hydrolyzed completely with 1 ml of 2 N trifluoroacetic acid at 121°C for 75 min. Half of the sugars released were reduced with 3 mg of NaBH₄ in 1 ml of 1 M NaOH for 1 h at room temperature, the reaction was stopped with glacial acetic acid and the borate was removed by addition of absolute methanol and evaporation to dryness at 60°C; these operations were repeated five times. The dried sample was resuspended in 2 ml of distilled water, and the radioactivity in 5 μl of solution was measured by scintillation spectrometry (Nuclear Chicago model 722 and 723). To obtain a standard solution, 1 mg of glucose was reduced to glucitol with 2 mg of NaBH₄, in 1 ml of 1 M NaBH₄ for 1 h at room temperature. The reaction was stopped with glacial acetic acid. The borate was eliminated by repeated evaporation with methanol at 60°C, and the resulting material was dissolved in 1 ml of distilled water.

**Thin Layer Chromatography of the Sugar Alcohols**—The sugar alcohols obtained following Smith degradation, redox degradation, Smith degradation, reduction with NaBH₄, hydrolysis, and reduction with NaBH₄ were chromatographed at room temperature in a mixture of 1-butanol, acetic acid, and 0.1 M sodium phosphate, pH 5 (40:50:10, v/v), on 250-μm layers of Kieselguhr G (E. Merck, Darmstadt, West Germany) prepared in 0.1 M sodium phosphate, pH 5. Five microliters of the sample solution and 5 μl of a 125 microcurie solution of the glucitol [14C]glucose on the same plate, and chromatography was continued until the solvent reached the upper border of the plate. The plate was dried, and chromatography then was repeated as before, separating mannitol (which has the greater Rv) from glucitol (14) by approximately 3 mm.

After the plate was again dried, one-half of each streak was visualized by spraying with a 0.5% solution of KMnO₄ in 1 N NaOH. The other half of each streak was scraped from the thin layer plate, and its radioactivity was determined by scintillation spectrometry.

**Sequential Degradation of the Backbones by Periodate Oxidation of Their Reducing-ends Sugars**

The 25 mg of backbone polysaccharide remaining from the first periodate oxidation were again subjected to Smith degradation. The process was the same as described above, except that the oxidation reaction was run for only 1 h in order to avoid overoxidation. The reaction was stopped with a few drops of a 0.1 M solution of sodium ascorbate (Na₂Asc), and the absence of periodate was determined with KI starch reagent (1). The solution was dialyzed, the solution was rechallenged with NaBH₄, and subjected to mild acid hydrolysis, and the solution then concentrated to 3 ml and lyophilized. The white residue was separated into two 2-mg portions, which were individually subjected to reducing-end analysis with NaBH₄, as described above, and into a residual portion (20 mg), which was used for the next Smith degradation. The process was repeated one additional time.

**RESULTS**

**Degradation by Glycosidases**—Of the glycosidases used, only the β-glucosidase could partially degrade the intact polysaccharides; and after it had acted, only the α-galactosidase could act on the residual polysaccharides. Only the β-mannosidase-containing β-mannanase S339Q could then partially degrade the remaining polysaccharides, followed by the α-glucosidase, and finally by the β-xylanase. Each enzyme released only a single monosaccharide, in amounts which were the same, within experimental error, for the polysaccharides from the envelopes of heterocysts and spores (Table I).

The results of methylation analysis of the polysaccharides remaining after each glycosidase reaction are presented in Table II. The figures are presented as moles per 4 mol of 3-linked sugars present in the molecules. The backbones, consisting of repetitions of 3-linked tetrasaccharides (1), remained intact. The results obtained with the polysaccharides from heterocysts and spores did not differ significantly.

**β-Glucosidase released only glucose; 4-glucose essentially disappeared, but the amount of T-glucose remained essentially invariant. α-Galactosidase released galactose, with extensive concomitant decrease in T-galactose and 3,6-glucose, and a corresponding increase in 3-glucose. Upon treatment with β-mannosidase-containing β-mannanase, mannose was released, T-mannose and 2,3-mannose virtually disappeared,**

1 We denote each partially methylated alditol acetate derivative by indicating the linkages (in addition to C-1) by which the corresponding glycosyl residue of the original sample was connected to other sugars. Thus, we write 4-glucose (4-Glc) instead of 1,4,5-tri-O-acetyl-2,3,6-tri-O-methyl galactitol. Partially methylated alditol acetate derivatives which were obtained from sugar derivatives that occupied nonreducing terminal positions in the polysaccharides, that is, which were glycosidically linked to other sugar residues only at C-1, are denoted by 2,3-di-O-acetyl-2,3,4,6-tetra-O-methyl galactitol is denoted T-galactose (T-Gal).

---

**TABLE I**

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Sugar released</th>
<th>Amount released from polysaccharide derived from</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Heterocyst</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-Glucosidase</td>
<td>Glucose</td>
<td>0.110</td>
</tr>
<tr>
<td>α-Galactosidase</td>
<td>Galactose</td>
<td>0.085</td>
</tr>
<tr>
<td>β-Mannosidase-contain-</td>
<td>Mannose</td>
<td>0.080</td>
</tr>
<tr>
<td>ing β-mannanase</td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-Glucosidase</td>
<td>Glucose</td>
<td>0.150</td>
</tr>
<tr>
<td>β-Xylosidase</td>
<td>Xylose</td>
<td>0.058</td>
</tr>
</tbody>
</table>

---

**TABLE II**

<table>
<thead>
<tr>
<th>Sugar released</th>
<th>Amount released from polysaccharide derived from</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>0.110</td>
</tr>
<tr>
<td>Galactose</td>
<td>0.085</td>
</tr>
<tr>
<td>Mannose</td>
<td>0.080</td>
</tr>
<tr>
<td>Xylose</td>
<td>0.058</td>
</tr>
</tbody>
</table>

---

**RESULTS**

**Degradation by Glycosidases**—Of the glycosidases used, only the β-glucosidase could partially degrade the intact polysaccharides; and after it had acted, only the α-galactosidase could act on the residual polysaccharides. Only the β-mannosidase-containing β-mannanase S339Q could then partially degrade the remaining polysaccharides, followed by the α-glucosidase, and finally by the β-xylanase. Each enzyme released only a single monosaccharide, in amounts which were the same, within experimental error, for the polysaccharides from the envelopes of heterocysts and spores (Table I).

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β-Glucosidase released only glucose; 4-glucose essentially disappeared, but the amount of T-glucose remained essentially invariant. α-Galactosidase released galactose, with extensive concomitant decrease in T-galactose and 3,6-glucose, and a corresponding increase in 3-glucose. Upon treatment with β-mannosidase-containing β-mannanase, mannose was released, T-mannose and 2,3-mannose virtually disappeared,
The sugars remaining after each step of the degradation are presented as moles per 4 mol of 3-linked sugars, i.e. relative to one repeating unit of the backbone. Whenever the amount of a particular derivative showed a conspicuous decrease or increase relative to the mean was less than the mean was less than 0.1 mol/mol of repeating unit.

### Table II

<table>
<thead>
<tr>
<th>Last enzyme to have acted</th>
<th>Source of polysaccharide</th>
<th>Sugar derivative</th>
</tr>
</thead>
<tbody>
<tr>
<td>None (intact polysaccharides)</td>
<td>H</td>
<td>T-Xyl</td>
</tr>
<tr>
<td>β-Glucosidase</td>
<td>H</td>
<td>0.30</td>
</tr>
<tr>
<td></td>
<td>S</td>
<td>0.40</td>
</tr>
<tr>
<td>α-Galactosidase</td>
<td>H</td>
<td>0.24</td>
</tr>
<tr>
<td></td>
<td>S</td>
<td>0.46</td>
</tr>
<tr>
<td>β-Mannosidase-containing β-mannanase</td>
<td>H</td>
<td>0.27</td>
</tr>
<tr>
<td></td>
<td>S</td>
<td>0.50</td>
</tr>
<tr>
<td>α-Glucosidase</td>
<td>H</td>
<td>0.36</td>
</tr>
<tr>
<td></td>
<td>S</td>
<td>0.31</td>
</tr>
<tr>
<td>β-Xylosidase</td>
<td>H</td>
<td>0.43</td>
</tr>
<tr>
<td></td>
<td>S</td>
<td>0.39</td>
</tr>
</tbody>
</table>

Values are means from double gas chromatograms from samples from each of three sequential digestions. The standard deviations averaged 3.4% of the mean values shown, except in those cases where the mean was less than 0.1 mol/mol of repeating unit.

and 3-mannose increased extensively, 2,3,4-Glucose disappeared, with release of glucose, upon subsequent treatment with α-glucosidase. There was an extensive concomitant decrease in T-glucose and an equivalent increase in 3,4-glucose. Finally, β-xylosidase, which released xylose, caused a disappearance of T-xylose, a corresponding decrease in 3,4-glucose, and a corresponding increase in 3-glucose. The remaining T-glucosyl residues could not be removed from the intact backbones with either α- or β-glucosidase.

**Cleavage of the Partially Degraded Polysaccharide by β(1 → 3)Endoglucanase and Analysis of the Products of Cleavage**—After partial degradation of the polysaccharides by β-glucosidase, α-galactosidase, β-mannosidase-containing β-mannanase, and α-glucosidase, the residual molecules consisted of β(1 → 3)-linked backbones with T-xylosyl and T-glucosyl side branches. Incubation of these swollen but still insoluble molecules with β(1 → 3)endoglucanase for 18 h released 60% of the end group equivalents (incubation of laminar with that enzyme for 18 h released 95% of the end group equivalents). The resulting clear solution was fractionated by passage through a column of Bio-Gel P-2 into a monosaccharide fraction, a trisaccharide fraction, and a fraction containing an oligosaccharide of higher molecular weight (Fig. 1). Per milligram of partially degraded polysaccharide digested, the amounts of each of these fractions obtained, as estimated by the anthrone test, were 0.26, 0.25, and 0.34 mg for the heterocyst polysaccharide, 0.28, 0.27, and 0.35 mg for the spore polysaccharide, respectively. During 18 h of electrophoresis, the trisaccharide from each cell type ran as a single spot with an electrophoretic mobility less than that of maltotriose, and the other oligosaccharide moved 1.5 cm from the origin, as a single spot.

Gas-liquid chromatography showed that the monosaccharide consisted only of glucose and that the trisaccharide contained mannose and glucose in the apparent ratio 1:1.7 (heterocyst-derived) or 1:1.9 (spore-derived). The larger oligosaccharide contained mannose, xylose, and glucose in the ratio 1:2:2.5 (heterocyst-derived) and 1:2:3.2 (spore-derived). Methylation analysis then showed that the trisaccharides contained T-glucose, T-mannose and 3,4-glucose in equal ratio (Fig. 2a). The larger oligosaccharides were shown to contain equal proportions of T-xylose, T-glucose, and 3-mannose, 4-glucose, and 3,4-glucose (Fig. 2b) and were therefore pentasaccharides.

α-Glucosidase was inactive on the trisaccharides, and β-glucosidase from almonds released only glucose and one-third of the reducing group equivalents. The same P-glucosidase, acting on the pentasaccharides, released only glucose and one-third of the reducing-end equivalents, and a tetrasaccharide.

The course of sequential degradation of the envelope polysaccharides from heterocysts (H) and spores (S) was estimated by the anthrone test, were 0.26, 0.25, and 0.34 mg for the spore polysaccharide, respectively. During 18 h of hydrolysis with NaBH₄, chromatography, localization of mannotol and glucitol, and elution from the thin layer plate, nearly
Fig. 2. Gas chromatographs of the partially methylated alditol acetate derivatives obtained (a) from the trisaccharide and (b) from the larger oligosaccharide formed by the action of β(1 → 3)endoglu.
canase S176N on the partially degraded polysaccharide formed from the envelope polysaccharide of heterocysts (see Fig. 1). Equivalent results were obtained with the spore-derived oligosaccharides. O-
Acetyl inositol was included as an internal standard. The glass chromatographic column used (2-mm inside diameter × 1.22 m) contained, on a stationary phase of Gas-chrom Q (80 to 100 mesh), 0.2%
poly(ethylene glycol adipate), 0.2% poly(ethylene glycol succinate), and 0.4% silicone GE-X560. This column was held at 110°C for 6 min
and then was temperature-programmed at 1°C/min from 110-190°C,
with a helium flow rate of 45 ml/min.

TABLE III
Radioactivity in glucitol and mannitol derived from glucose and
mannotose at the reducing terminus of backbone polysaccharides
formed from the envelope polysaccharides of heterocysts and
spores of A. cylindrica which were subjected repeatedly to Smith
degradation

<table>
<thead>
<tr>
<th>Times oxidized with periodate</th>
<th>Counts per min/5 μg of polysaccharide derived from</th>
<th>Heterocysts</th>
<th>Spores</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Glucitol</td>
<td>Mannitol</td>
<td>Glucitol</td>
</tr>
<tr>
<td>1</td>
<td>1150 ± 50</td>
<td>12 ± 6</td>
<td>1350 ± 48</td>
</tr>
<tr>
<td>2</td>
<td>1185 ± 35</td>
<td>15 ± 8</td>
<td>1225 ± 30</td>
</tr>
<tr>
<td>3</td>
<td>10 ± 5</td>
<td>1000 ± 30</td>
<td>6 ± 5</td>
</tr>
</tbody>
</table>

all of the radioactivity was associated with glucitol: 1150 ± 50
cpm for the heterocyst-derived polysaccharide and 1350 ± 48
cpm for the spore-derived polysaccharide (30.5 nmol of hexose,
in each case). [3H]Glucitol, derived from the standard solution of
glucose and diluted 100-fold, had a radioactivity of 1550 ±
50 cpm/0.278 nmol. Upon oxidation of each backbone with
periodate, the terminal sugar was again glucose. After an
additional oxidation, the terminal sugar was mannotose (Table
III).

DISCUSSION
We propose that the polysaccharides from the envelopes of
heterocysts and spores of A. cylindrica consist of combinations of skeletal subunits of the form

Glc
\[ \text{Man} \rightarrow \text{Glc} \rightarrow \text{Glc} \rightarrow \text{Glc}, \]

Glc
to which additional sugar residues may be added, approxi-
mately as shown in Fig. 3. The following considerations lead
us to make these proposals.

1. Treatment of the native polysaccharides with β-glucosi-
dase results in the loss of 4 linked glucosyl residue from the
polymers without corresponding changes in the amounts of
T-glucosyl residues or of residues of any branched sugars
(Table II). We conclude that the β-glucosidase hydrolyzes T-

-glycosyl residues that are β-linked to position C-4 of glucose
in a di- or oligosaccharide side chain. An average of 1 T-

-glucosyl residue is removed per tetrasaccharide repeating unit
(1) of the backbone.

2. Subsequent treatment with α-galactosidase results in the
loss of T-galactosyl and 3,6-glucosyl residues, with a corre-
sponding increase in the amount of 3-glucosyl residues (Table
II). We conclude that the T-galactosyl residues are α-linked
as monosaccharide side chains to C-6 of 3,6-linked glucosyl
residues in the polymers.

3. Treatment of the residual polysaccharides with the β-
mannosidase-containing β-mannanase results in the loss of T-

-mannosyl and 2,3-mannosyl residues, with a corresponding
increase in the amount of 3-mannosyl residues. We conclude
that the T-mannosyl residues are β-linked as monosaccharide
side chains to C-2 of the 2,3-linked mannosyl residues in the
polymers.

Fig. 3. Approximate subunit structure of the polysaccharides from
the envelopes of heterocysts and spores of A. cylindrica. Linkages to
T-xylose, T-galactose and T-mannotose indicated by dashed lines are
present attached to only about half of the (residual) units shown. The
terminal sugar of the disaccharide branch may in fact be linked to C-
4 of the glucose linked to C-2 of the same glucosyl residue of the
backbone, and T-galactose may possibly be attached to some of those

-glucosyl residues of the backbone, at the reducing end of the repeating
unit shown, to which xylosyl residues are not attached. Unlinked
carbon atoms 6 are omitted from the drawing for the sake of simplifi-
cation.
Heterocyst and Spore Polysaccharides

4. Treatment of the residual polysaccharides with α-glucosidase results in the loss of T-glucose and 2,3,4-glucosyl residues with a corresponding increase in the amount of 3,4-glucosyl residues. We conclude that T-glucosyl residues are α-linked as monosaccharide side chains to C-2 of the 2,3,4-linked glucosyl residues in the polymers.

5. Treatment of the remaining polysaccharides with β-xylosidase results in the loss of T-xylosyl and 3,4-glucosyl residues and a corresponding increase in the amount of 3-glucosyl residues. We conclude that the T-xylosyl residues are β-linked as monosaccharide side chains to C-4 of the 3,4-glucosyl residues present in these polymers.

6. Because the T-galactosyl, T-mannosyl, and T-xylosyl side branches account for the 3,6-glucosyl, 2,3-mannosyl and 3,4-glucosyl branch points in the backbones, respectively (see above), we further conclude that the T-glucosyl residues hydrolyzed by β-glucosidase were linked to glucose residues present at position C-2 or C-4 of the 2,3,4-glucosyl residues. Attempts by means of partial acid hydrolysis of the intact polysaccharides to remove glucosyl-glucosyl branches as a whole, or to leave them attached while removing the T-glucosyl branches, were unsuccessful. It therefore remains unknown which of these branches were present at position C-2, and which at position C-4, of the 2,3,4-glucosyl residues.

The β(1→3)endoglucanase S176N is capable of hydrolyzing the backbones of the polysaccharides from the envelopes of heterocysts and spores (1). It can still do so when T-glucose (at C-4 of the original 2,3,4-glucosyl residues) and T-xylose are attached to the backbones. The products of action of the endoglucanase are glucose, the trisaccharide,

\[
\text{Glc} \quad \text{Man} \quad \text{Glc}
\]

and a pentasaccharide which, according to methylation analysis, could be either

\[
\begin{align*}
\text{Xyl} & \quad \text{Glc} \\
1 & \quad 4 \\
\text{Glc} & \quad \text{Man} \quad \text{Glc}
\end{align*}
\]

or

\[
\begin{align*}
\text{Glc} & \quad \text{Xyl} \\
1 & \quad 4 \\
\text{Glc} & \quad \text{Man} \quad \text{Glc}
\end{align*}
\]

(The Exo-β-glucosidase digestion of b should have resulted in the successive excision of 2 glucosyl residues, leaving a trisaccharide. However, the digestion of the pentasaccharide by β-glucosidase produced glucose and a tetrasaccharide. Thus, only structure a, which also accords with the structure of the trisaccharide, is consistent with the experimental results.

Cleavage of the partially digested polysaccharides by the β(1→3)endoglucanase did not produce free xylose or mannose and did not change the ratio (±1) of glucosyl(1→4)glucosyl linkages to mannosyl residues. Thus, all side branches remained, so that all branched residues remained. However, all unbranched glucosyl residues were released, as may be seen by comparison of the structures of the tri- and pentasaccharides with the structure shown in Fig. 3. It follows that the β(1→3)endoglucanase cleaved to both sides of all unsubstituted glucosyl residues in the backbones of the partially degraded polysaccharides.

As shown by the action of β-glucosidase on the tri- and pentasaccharides obtained by use of the β(1→3)endoglucanase, the glucosyl residues present at C-4 of the originally 2,3,4-linked glucosyl residues were β-linked. The fact that the glycosidases could act only in a particular sequence is presumably attributable to steric hindrance of their activity. The ability of the β-glucosidase to detach the T-glucosyl residues which were 1→4 linked to backbone glucosyl residues only after, but not before, endoglucanase digestion may, however, correspond to a requirement by this enzyme for longer linear chains as substrate (15).

The T-glucose in the tri- and pentasaccharides was initially attached to the 2,3,4-glucose of the original polysaccharides, the doubly branched sugars were to the reducing-end side of the mannosyl residues in the backbones of the original polysaccharides. Similarly, the T-xylose branches were linked to position C-4 of approximately half of the glucosyl residues linked to C-3 of those mannosyl residues. In the original polysaccharides, the T-galactosyl residues may be linked to approximately half of positions C-6 of the 3rd glucosyl residue of the repeating tetrasaccharide units of the backbones. Alternatively, the backbone glucosyl residue linked to C-3 of the mannosyl residues may be linked either through C-4 to xylose or through C-6 to galactose, with the center glucosyl residues in the backbones unbranched. Finally, only about half of the mannosyl residues in the backbones are linked through C-2 to a T-mannose.

Glucitol formed by reduction of the backbone polysaccharides derived from the envelopes of heterocysts and spores had a mean radioactivity of \(3.8 \times 10^5\) and \(4.4 \times 10^5\) cpm/μmol of polysaccharide hexose, respectively. Glucitol formed by reduction of glucose with NaB\(_4\)H\(_4\), had a radioactivity of approximately \(5.6 (±0.2) \times 10^6\) cpm/μmol of glucose. On the basis of the ratios of these specific activities, we conclude that the backbone polymers had a chain length of approximately 128 (spore-derived) to 150 (heterocyst-derived) hexose units, consistent with earlier, less exact determinations (1). The average molecular weight of the intact polysaccharide may therefore be calculated to be approximately 45,000.

Preliminary observations showed that the reducing terminus of polysaccharides in lipid-free envelopes was glucose. Smith degradation would have removed the reducing terminus of the polysaccharides as well as their side branches. After Smith degradation, and a repetition of it, the reducing terminus continued to be glucose. However, after the polysaccharides were subjected to Smith degradation for a third time, the sugar at the reducing end of the remaining backbones was mannose. Therefore, the backbones of the polysaccharides terminate in the sequence Man → Glc → Glc → Glc. Because it was established earlier that the backbones consist of repetitions of such a tetrasaccharide (1), we propose that the structure of the backbones is \((\text{Man} \rightarrow \text{Glc} \rightarrow \text{Glc} \rightarrow \text{Glc})_n\).

Whether the envelopes contain a mixture of slightly differing polysaccharides, each of which has a repeating subunit, or whether any given polysaccharide chain contains skeletal subunits which differ in the number of side branches which they bear, cannot at present be decided. Our previous (1) and present results have shown no significant differences between the structures of the polysaccharides from the envelopes of heterocysts and spores. These polysaccharides therefore appear to be essentially identical, and identity of structures of such complexity implies identity of the processes which led to
their biosynthesis. We suggest that in analogy to the biosynthesis of peptidoglycan (2, 16) and of O-antigen (3, 17, 18), the skeletal subunit, possibly additionally branched, is first synthesized and then assembled as prefabricated units into the elongating envelope polysaccharides.

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