Heteroglycan Synthesis in Cryptococcus laurentii*

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SUMMARY

An enzyme preparation from Cryptococcus laurentii catalyzes the transfer of glycosyl units from sugar nucleotides to exogenous acceptor resulting in the stepwise synthesis of a heteroglycan with the following structure: Z-O-α-D-mannosyl-6-O-α-D-mannosyl-3-O-α-D-mannosyl-(2-O-β-D-xylosyl-)D-mannose. The same enzyme preparation transfers D-[14C]-mannosyl from GDP-[14C]mannose to an endogenous glycoprotein acceptor. Analysis of the endogenous product after mild acid hydrolysis revealed the presence of Man-2-mannose, Man-3-mannose, and Man-6-mannose. The enzymes synthesizing the specific mannosyl linkages in the pentasaccharide have been shown to be distinct mannosyltransferases (SCHUTZBACH, J. S., AND ANKEL, H. (1972) J. Biol. Chem. 246, 2187–2194). The β-xylosyl linkage in the pentasaccharide is made by an enzyme that also transfers xylose from UDP-xylose to the same endogenous acceptor (SCHUTZBACH, J. S., AND ANKEL, H. (1972) J. Biol. Chem. 247, 6574–6580). Glycosyl transfer to exogenous and to endogenous acceptors appears to involve the same enzymes, suggesting that the pentasaccharide synthesized de novo is an integral part of the cell envelope glycoprotein.

The structure and biosynthesis of the carbohydrate chains of glycoproteins have come under intensive investigation in recent years. These carbohydrate chains are often composed of more than one sugar and are quite complex in structure (1). The biosynthesis of these oligosaccharides is believed to proceed by the stepwise addition of individual sugars to the growing chain from the corresponding sugar nucleotides (1, 2). This is in contrast to the biosynthesis of many microbial polysaccharides that are synthesized from a second type of activated glycosyl residue, linked through a phosphodiester or a pyrophosphate linkage to polysaccharide lipid (3).

We would now like to report the synthesis of a branched chain heteropentasaccharide by enzymes from the fungus imperfectus Cryptococcus laurentii. The pentasaccharide contains four α-D-mannosyl residues and a terminal branched β-xylosyl residue and is similar in structure to the cell envelope glycoprotein complex of the organism. The stepwise biosynthesis and the structural analysis of this pentasaccharide are described in this paper.

EXPERIMENTAL PROCEDURES

Materials

Man-2-mannose, Man-3-mannose, and Man-6-mannose were prepared as previously described (4, 5). Sweet almond Emulsin (Warburg) was used as a source of α-mannosidase (6, 7). All other chemicals were obtained from commercial sources.

Enzyme Preparation

C. laurentii var. flavescens (NRRL Y-1401) was grown and harvested as described previously (8). The standard medium consisted of 0.5% glucose (w/v), 0.1% yeast, 0.1%KH2PO4, 0.05% MgSO4.7H2O, and 0.2 mg per liter of thiamine.HCl. The preparation of cell-free extracts and the isolation of the particulate enzyme fraction followed procedures previously described (6, 6). All operations were carried out at 0–4°C. Log phase cells (A at 400 nm, 0.7) were harvested by centrifugation at 1,000 g for 15 min, washed with 10 volumes of 1% NaCl, 10 volumes of distilled water, and 10 volumes of 0.1 M Tris-HCl buffer, pH 7.3, 1 mM in reduced glutathione and EDTA. The washed residue was resuspended in approximately 1 volume of the same buffer and was disrupted by ultrasonic treatment in a Branson Sonifer (model 125) for 6 min at an output of 11 amperes.

The cell debris was removed by centrifugation at 12,000 g for 15 min and the particulate fraction was then isolated by centrifugation at 105,000 g for 30 min. The pellet so obtained was resuspended in approximately 10 volumes of the above buffer and again centrifuged. This step was repeated once. The particulate material separate into three distinct layers. The brown middle layer contains all of the glycosyltransferase activities and was carefully isolated and resuspended in 3 volumes of the same buffer. The final pH of the enzyme suspension is approximately 4.5 to 5.5 and the buffering capacity of the protein maintains this pH in unbuffered reaction mixtures. The enzymes retain activity for at least 2 weeks at 4°C.

Analytical Methods

Protein was determined by the procedure of Lowry et al. (9). Radioactivity was measured in a gas flow counter (Nuclear-Chicago) or in a liquid scintillation counter (Packard) using Bray's solution (10) or toluene scintillation fluid (Spectrafluor, American-Searle). Paper chromatograms and electrophoretograms were analyzed for radioactivity with a Nuclear-Chicago Actigraph III or by autoradiography.

Chromatography and Electrophoresis

Paper chromatography was carried out on Whatman No. 1 or 3MM paper. The solvents used were: (a) 1-propanol-ethyl ace-

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1 The abbreviations used are: Man-2-mannose, 2-O-α-D-mannosyl-D-mannose; Man-3-mannose, 3-O-α-D-mannosyl-D-mannose; Man-6-mannose, 6-O-α-D-mannosyl-D-mannose; Man-CH3, methyl-α-D-mannopyranoside; β-Xyl-2-mannose, 2-O-β-D-xylosyl-D-mannose.

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tate-water, 7:1:2; (b) ethyl acetate-pyridine-water, 8:2:1; (c) ethyl acetate-acetic acid-water, 5:1:1; (d) 95% ethanol-1 M ammonium acetate 7:3; (e) ethyl acetate-pyridine-water, 70:25:5; (f) ethyl acetate-1-propanol-water, 5:3:2.

Electrophoresis was carried out on Whatman No. 1 paper at 30 volts per cm for 5 hours in 0.04 M sodium borate, pH 9.2. Carbohydrates were detected with p-anisidine phthalate or by silver nitrate-acetone followed by alcoholic sodium hydroxide spray (11).

Reduction with NaBH₄

Reductions were carried out by adding 0.05 ml of 0.5 M NaBH₄ to a solution of radioactive sugars in 0.1 ml of water. The reaction mixtures were incubated at room temperature for 16 hours, then treated with cation exchange resin. Boric acid was removed by repeated evaporation in the presence of methanol.

Acetolysis

This was carried out essentially as described by Stewart et al. (12), but the time of acetolysis was changed to 16 hours and the temperature to 35°. Under these conditions, optimal amounts of disaccharide appeared to be formed. After methanolysis the acetolysis products were separated in Solvent 1.

Preparation of Methyl-trisaccharide

We have shown that the particulate “middle layer” enzyme preparation from C. laurentii transfers mannose from GDP-mannose to Man-CH₂ in a Mg²⁺-dependent reaction to form Man-3-Man-CH₂ (5). A second enzyme in the preparation catalyzes the transfer of D-xylose from UDP-xylose to Man-3-mannose and to oligosaccharides containing this structure at the terminal non-reducing end (5). The latter reaction is metal ion-independent and the product contains a branched D-xylosyl residue linked in a β-1,2-linkage to the penultimate α-mannosyl residue. It also transfers D-xylose to Man-3-Man-CH₂ to form a branched methyl-trisaccharide (5).

We have combined these two reactions to prepare methyl-trisaccharide labeled with [14C] in either a β-mannosyl or in the D-xylosyl residue.

Procedure 1—Reaction mixtures containing Man-CH₂ (80 nmole), GDP-[¹⁴C]mannose (0.35 µCi; 2.1 pmole), UDP-xylose (1.3 µmol), MgCl₂ (12 µmol), and enzyme (5 µg of protein) in a total volume of 0.5 ml of 0.1 M Tris-acetate buffer, pH 7.0 (buffer) were incubated for 20 hours at 25°. Particulate material was removed by centrifugation at 9000 X g for 15 min. Chromatography of the supernatants in Solvent 1 revealed the formation of a radioactive product (1.03 pmole) with the mobility of methyl-trisaccharide (Fig. 1). The product was eluted and used as an acceptor for the following experiment.

Procedure 2—A [¹⁴C]xylosyl labeled product (0.11 µCi; 0.67 pmole; Fig. 1) was prepared in a similar reaction mixture with UDP-[¹⁴C]xylose (0.2 µCi; 1.3 pmole) of GDP-[¹⁴C]mannose. The methyl-trisaccharide was not formed if GDP-mannose, UDP-xylose, or MgCl₂ were omitted from the reaction mixtures.

RESULTS

The particulate “middle layer” enzyme preparation from C. laurentii contains enzymes that catalyze the stepwise synthesis of the branched trisaccharide α-Man-1,3-(β-Xyl-1,2)-Man-CH₂ (5, 6). We would now like to demonstrate that this trisaccharide is an acceptor for a second mannosyltransferase resulting in the formation of a methyl-tetrasaccharide. The latter product is in turn an acceptor for a specific mannosyltransferase with formation of a methyl-pentasaccharide. We would also like to present evidence that the glycosyl-linkages present in the pentasaccharide are found in cell envelope glycoproteins synthesized by the organism.

Synthesis of Methyl-tetrasaccharide and Methyl-pentasaccharide—Sixty nanomoles of UDP-[¹⁴C]xylose labeled methyl-trisaccharide prepared according to Procedure 2 was incubated with GDP-[¹⁴C]mannose (0.1 µCi; 0.65 pmole), MnCl₂ (1 µmol), and enzyme (1.5 mg of protein) in a total volume of 0.15 ml of buffer for 18 hours at 25° (Procedure 3). The reaction mixture was applied to paper and the reaction products were separated by chromatography in Solvent 1. As can be seen in Fig. 2B, a new product was formed. When MnCl₂ was omitted from the reaction mixture or was replaced with 1 µmol of MgCl₂, the product was not formed.

In an attempt to increase the yield of the new product, a presumed methyl-tetrasaccharide, a similar reaction mixture was prepared except that the GDP-[¹⁴C]mannose concentration was increased to 0.6 µmol (0.1 µCi). Chromatography of the reaction mixture (Fig. 2C) revealed that a product with a still slower chromatographic mobility had been synthesized. It was assumed that in the absence of excess GDP-mannose, 2 β-mannosyl residues were added to the trimer resulting in the formation of a methyl-pentasaccharide. The following experiment was carried out in order to confirm the degree of polymerization of the higher oligomers.

A substrate mixture containing UDP-[¹⁴C]xylose (0.25 µCi; 0.27 µmole) and GDP-[¹⁴C]mannose (0.3 µCi; 0.25 µmole) in
0.1 ml of water was prepared. Fifty microliters of this substrate mixture were incubated with Man-CH₃ (25 μmoles), enzyme (7.5 mg of protein), and MgCl₂ (10 μmoles) in a total volume of 0.45 ml of buffer for 18 hours at 25°. The [¹⁴C]mannosyl-[¹⁴C]-xylosyl-labeled trisaccharide formed (0.05 μCi) was isolated after chromatography and was then incubated with the substrate mixture prepared above (50 μl), enzyme (7.5 mg of protein), and MnCl₂ (5 μmoles) in a total volume of 0.45 ml of buffer at 25°. After 18 hours the reaction mixture was subjected to chromatography in Solvent 4 and the radioactive products were located by autoradiography. Radioactive bands were eluted and purified by chromatography in Solvent 1. The only radioactive compounds detectable had the chromatographic mobilities expected for methyl-trisaccharide, methyl-tetrasaccharide, and methyl-pentasaccharide with the chromatographic mobilities expected for methyl-trisaccharide, methyl-tetrasaccharide, and methyl-pentasaccharide.

After 18 hours the reaction mixture was subjected to chromatography and was then incubated with the substrate mixture prepared above (50 μl), enzyme (7.5 mg of protein), and MnCl₂ (5 μmoles) in a total volume of 0.45 ml of buffer at 25°. After 18 hours the reaction mixture was subjected to chromatography in Solvent 4 and the radioactive products were located by autoradiography. Radioactive bands were eluted and purified by chromatography in Solvent 1. The only radioactive compounds detectable had the chromatographic mobilities of authentic n-mannose and n-xylose. The radioactive sugars were eluted in a toluene scintillation mixture, and the [¹⁴C]mannose to [¹⁴C]xylose ratio was determined (Table I). The data show that the ratios of label indicate that the products isolated were in fact methyl-trisaccharide, methyl-tetrasaccharide, and methyl-pentasaccharide.

In order to determine the metal requirement for the conversion of tetramer to pentamer, labeled methyl-tetrasaccharide was prepared according to Procedure 3. Labeled tetramer (0.01 μCi; 0.055 nmole) was then incubated with GDP-mannose (0.1 μmol) and enzyme (0.5 mg of protein) for 18 hours at 25° in reaction mixtures of 0.06 ml of buffer in the absence and in the presence of 1 μmol of either MnCl₂ or MgCl₂. Only in the mixtures containing MnCl₂ was the methyl-pentasaccharide formed (0.065 nmole) as judged after chromatography of the reaction mixtures in Solvent 1.

### Analysis of Higher Oligomers

In order to facilitate description of the position of the mannosyl linkages in the methyl-pentasaccharide we will refer to the numbers given in Fig. 3.

Methyl-tetrasaccharide containing n-[¹⁴C]mannose at Position II was prepared from trimer synthesized according to Procedure 1. The labeled methyl-trisaccharide (0.26 μmol) was isolated and incubated with unlabeled GDP-mannose (0.44 μmol), enzyme (5 mg of protein), and MnCl₂ (10 μmoles) in 0.4 ml of buffer at 25° for 6 hours. The reaction mixture was subjected to chromatography in Solvent 4 and methyl-tetrasaccharide (60 nmol) and methyl-pentasaccharide (151 nmol) were isolated and further purified by chromatography in Solvent 1. Limited hydrolysis of the isolated pentamer with 1 mg of Emulsin in 0.2 ml of 0.5 M ammonium acetate, pH 5.0, for 18 hours preferentially removed the terminal n-mannosyl and allowed isolation of more tetramer. Both tetramers gave identical results when the mannosyl linkages were characterized as described below.

The methyl-[¹⁴C]tetramer isolated above (60 nmol) was hydrolyzed in 0.1 M HCl for 80 min at 100° and the hydrolysate was analyzed by chromatography in Solvent 1 (Fig. 4A). The disaccharide unit from the reducing terminus, [¹⁴C]Man-3-mannose, was isolated in 2% yield based on [¹⁴C]-counts. A second disaccharide with the mobility of Man-4-mannose was isolated in 7% yield and shown to be Man-6-[¹⁴C]mannose. The disaccharide was quantitatively hydrolyzed by α-mannosidase when treated with two additions of 1 mg of Emulsin for 48 hours in 0.1 M ammonium acetate, pH 5.0. Chromatography of the hydrolysate in Solvent 1 showed n-[¹⁴C]mannose to be the only labeled product. The disaccharide comigrated with authentic Man-6-mannose in Solvents 1 and 3. These procedures clearly separate Man-6-mannose from the other α-linked mannobioses (6). The labeled α-1,6-linked disaccharide was reduced with NaBH₄, hydrolyzed in 0.1 M HCl for 2 hours at 100°, and the hydrolysate was analyzed by chromatography in Solvent 2. Man-6-mannose isolated from methyl-tetrasaccharide labeled at position II yielded only [¹⁴C]mannitol after reduction and hydrolysis (Fig. 5A) showing that the labeled sugar was at the reducing end. Further chromatography in Solvent 5 showed that the [¹⁴C]-labeled material again comigrated with authentic mannitol.

These results show that mannosyl was transferred from GDP-mannose to the [¹⁴C]mannosyl of the methyl-trisaccharide with formation of an α-1,6-linkage. In order to provide additional evidence for the synthesis of the α-1,6-linkage, methyl-tetrasaccharide containing [¹⁴C]mannosyl at position III was prepared according to Procedure 3. The [¹⁴C]-labeled tetramer was

![Fig. 3. The structure of the methyl-pentasaccharide. All mannosyl linkages are alpha and the numbers refer to consecutive mannosyl residues starting at the potential reducing end of the oligosaccharide.](http://www.jbc.org/)

### Table I

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<td>1.42</td>
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<td>0.9:1.0</td>
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<td>2.30</td>
<td>1.13</td>
<td>2.0:1.0</td>
<td>4</td>
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<tr>
<td>III</td>
<td>1.51</td>
<td>0.52</td>
<td>2.9:1.0</td>
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hydrolyzed in 0.1 N HCl as above and the hydrolysate was subjected to chromatography in Solvent 1 (Fig. 4B). The only $^{14}$C-labeled disaccharide that could be detected had the mobility of Man-6-mannose and this was eluted from the paper in a yield of 8% based on radioactivity. This disaccharide was hydrolyzed by $\beta$-mannosidase when treated as above and had the mobility of Man-6-mannose in Solvents 1 and 3. The disaccharide was reduced with NaBH$_4$, hydrolyzed, and analyzed as above. All of the label was recovered as $\beta$-[C]mannose, showing that this disaccharide was [C]Man-6-mannose.

These results indicate that the structure of the methyl-tetrasaccharide is: Man-6-Man-3-$\beta$-Xyl-2-Man-CH$_3$.

Additional evidence for the presence of the $\alpha$-1,6-linkage is the lability of this linkage upon acetolysis according to the procedure of Stewart et al. (12). This fact enabled isolation of the terminal nonreducing mannobiose unit from the methyl-pentasaccharide. Methyl-tetrasaccharide containing [C]mannosyl at position III was prepared according to Procedure 3. The [C]tetramer (0.6 nmole) was isolated as before and incubated with enzyme (5 mg of protein) in reaction mixtures containing GDP-mannose (0.6 $\mu$ mole) and MnCl$_2$ (0.4 $\mu$ mole) in a total volume of 0.4 ml of buffer for 18 hours at 25°. Carbon-14 labeled methyl-pentasaccharide (0.6 nmole) was separated by chromatography of the reaction mixture in Solvent 1. The pentamer was subjected to acetolysis and the products were separated by chromatography in Solvent 1. Analysis of the chromatogram by autoradiography revealed that a disaccharide with the mobility of Man-2-mannose was liberated in good yield (0.18 nmole, 29%). The disaccharide was eluted and characterized. Treatment with $\alpha$-mannosidase as before resulted in complete hydrolysis with the liberation of free n-[C]mannose and 4 n-mannose, the other with the mobility of Man-6-mannose. The structure of Man-2-mannose was confirmed by electrophoresis in borate buffer and hydrolysis by Emulsin. These results indicate that the last mannosyl residue added to the pentamer must be bound in an $\alpha$-1,2-linkage to the n-mannosyl at position III (see Fig. 3). The complete structure of the methyl-pentasaccharide is given in Fig. 3.

The methyl group at the reducing end of the oligosaccharide is not necessary for the synthesis of a pentasaccharide. Starting with Man-3-mannose instead of Man-CH$_3$ as the acceptor, we have found that reaction mixtures as above described lead to the stepwise synthesis of a branched pentasaccharide with a free reducing end. This product too contains 4 n-mannose and 1 $\beta$-xylose and presumably has the same structure as the methyl derivative analyzed above. Indeed, it is possible to isolate...
Man-2-mannose and Man-6-mannose from partial acid hydrolysates of this product. The more thorough characterization of the oligosaccharide structure was carried out with the methyl derivative because of the faster chromatographic mobilities of the methyl-saccharides as compared to the free saccharides and the higher yield of specifically [14C]mannosyl-labeled products that could be obtained.

Attempts to synthesize oligosaccharides of even higher molecular weight by the stepwise addition of glycosyl units have been made but the results have been negative. Carbon-14 labeled methyl-pentasaccharide was incubated with enzyme in the presence of GDP-mannose, UDP-xylose, and UDP-galactose, either alone or in combination, and with a variety of metal ions including Mn2+, Mg2+, Zn2+, and Co2+. Analysis of the reaction mixtures by chromatography in Solvent 1 failed to reveal the formation of oligomers with a degree of polymerization greater than five.

**Mannosyl Transfer to Endogenous Acceptor**—We have previously demonstrated the transfer of α-mannose from GDP-[14C]mannose to an endogenous acceptor in the middle layer (4). The reaction required the addition of divalent metal ions for activity, Mn2+ being most effective. Analysis of the reaction product formed in the presence of MnCl2 by acetolysis showed that two different mannosyl-mannose linkages were formed, Man-2-mannose and Man-3-mannose. The following experiments were designed to identify α-1,6-mannosyl-mannose linkages in the endogenous product and to relate synthesis of this heteroglycan to specific mannosyltransferases.

Reaction mixtures containing enzyme (12.5 mg of protein), GDP-[14C]mannose (1 µCi; 0.5 nmoles), and either MnCl2 (5 µmoles) or MgCl2 (10 µmoles) in a total volume of 0.8 µl of 0.1 M Tris-acetate buffer, pH 7.5, were incubated at 25°C. After 18 hours, the product was isolated by centrifugation at 9000 x g for 15 min. The precipitate was washed five times with 0.5 ml of water and recovered each time by centrifugation as above. The Mn2+ product (0.23 µCi of [14C]mannosyl) and the Mg2+ product (0.2 µCi of [14C]mannosyl) were dissolved in 1 ml of 4% (w/v) sodium dodecyl sulfate. Insoluble material was removed by centrifugation as above and all of the counts were recovered in the supernatant. Five volumes of 95% ethanol were added to the supernatant and after 2 hours at 2°C the precipitates, containing all of the label, were recovered by centrifugation. The precipitates were resuspended in 2 ml of water, 5 mg of pronase (Calbiochem) were added, and the mixtures were incubated for 24 hours at 25°C. The Mn2+ product (0.18 µCi) and the Mg2+ product (0.16 µCi) were recovered in the supernatants and further analyzed.

**Analysis of Products**—Endogenous products as prepared above were dried under reduced pressure, dissolved in 1 ml of 5% sulfuric acid, and hydrolyzed at 80°C for 1 hour. The hydrolysates were neutralized with Ba(OH)2, the precipitates were removed by centrifugation at 9000 x g for 15 min, and the supernatants were again dried under reduced pressure. Chromatography in Solvent 1 revealed that free α-[14C]mannose was liberated along with a number of other radiolabeled products (Fig. 7).

Hydrolysates of the Mn2+ product contained labeled compounds that had chromatographic mobilities corresponding to Man-6-mannose and Man-2-mannose and (or) Man-3-mannose. The radioactive disaccharides were eluted and further purified by chromatography in Solvent 1. Complete hydrolysis of a sample of the isolated disaccharides in 1 N HCl for 2 hours at 100°C followed by chromatography in Solvent 1 revealed only the presence of α-[14C]mannose. [14C]-labeled α-mannose was also the only detectable product when the disaccharides were reduced with NaBH4 prior to the hydrolysis and the hydrolysates were analyzed by chromatography in Solvent 2. The disaccharides were completely hydrolyzed by an α-mannosidase under the conditions used before. The compound with the chromatographic mobility of Man-2-mannose was subjected to electrophoresis in borate buffer. Analysis of the electrophoretogram by autoradiography showed the presence of two different disaccharides with the mobilities of Man-2-mannose and Man-3-mannose. This is in agreement with our previous results obtained by acetylation of the endogenous [14C]mannosyl product formed in the presence of Mn2+ (4). The partial hydrolysate product with the mobility of [14C]Man-6-mannose was analyzed by chromatography in Solvents 1, 2, and 3. In each system the [14C]-labeled compound comigrated with authentic Man-6-mannose.

In contrast, partial hydrolysis of the Mg2+ product as above gave only a single mannotriose upon chromatography in Solvent 1 (Fig. 7). The disaccharide was analyzed as with the Mn2+ product and shown to contain a terminal α-linked [14C]mannosyl residue. [14C]Man was the product obtained after reduction with NaBH4, hydrolysis, and chromatography in Solvent 2. Electrophoresis in borate buffer identified the disaccharide as Man-3-mannose.

**DISCUSSION**

*C. laurentii* contains a neutral heteroglycan composed mainly of α-mannose, β-xylose, and β-galactose. This heteroglycan can be extracted with ethylene diamine from intact cells, from isolated cell wall fragments, and from the particulate preparation that contains the enzyme activities described in this paper (4). We have shown in previous work that mannosyl transfer from GDP-[14C]mannose to terminal nonreducing ends of an endogenous acceptor similar in composition and structure to...
the heteroglycan with the formation of α-1,2- and α-1,3-mannosyl-mannosyl linkages (4).

This publication presents evidence for the synthesis of α-1,6-mannosyl-mannosyl linkages and relates synthesis of the branched heteropenta saccharide described herein to distinct mannosyl- and xylosyltransferase activities and to the biosynthesis of the cell envelope heteroglycan.

C. laurentii contains four different mannosyltransferases, one of which catalyzes the transfer of β-mannose from GDP-mannose to Man-CH₂ with formation of Man-3-Man-CH₂ (6). The enzyme is activated by Mg²⁺, Mn²⁺, and other divalent metals, Mg²⁺ being most effective. Other mannosyltransferases present in the organism are not significantly activated by Mg²⁺. This Mg²⁺ activated transferase catalyzes synthesis of the Man-3-mannose linkage in the pentasaccharide and synthesis of an α-1,3 linkage in endogenous product. The endogenous [¹⁴C]mannosyl product formed in the presence of Mg²⁺ is very similar in properties to endogenous [¹⁴C]mannosyl product formed in the presence of Mn²⁺. This indicates that the α-1,3-linkage formed in the presence of Mn²⁺ is synthesized by the Mg²⁺ transferase.

The second transfer reaction involves addition of xylose to the Man-3-mannose unit and results in the formation of a branched product containing a β-1,2-Xyl-Man linkage (5). The same xylosyltransferase was shown by heat inactivation, inhibition by nucleotides, and by pH optimum to also transfer xylose to endogenous acceptor. The latter reaction was greatly stimulated in the presence of GDP-mannose and Mg²⁺, conditions favorable to the synthesis of Man-3-Man units in the acceptor.

A second mannosyltransferase catalyzes the formation of α-1,6-mannosyl-mannose and a third the formation of α-1,2-mannosyl-mannose linkages. The latter two enzymes require Mn²⁺ for activity and are practically inactive in the presence of Mg²⁺. Previous studies utilizing exogenous acceptors have shown these activities to be due to specific enzymes (6).

Our results suggest that the pentasaccharide structure described herein, 2-0-α-α-mannosyl-6-0-α-β-mannosyl-3-0-β-mannosyl-(2-0-β-β-xylosyl-) β-mannose, is an integral part of the cell envelope. The enzymes synthesizing the heteropenta saccharide also form the same linkages in endogenous polymers. Previous studies (4) have shown the presence of the α-1,2- and α-1,3-linked mannosyl disaccharide units in the endogenous polysaccharide but failed to reveal the presence of Man-6-mannosyl units. This can be explained by the fact that the neoglycosylation procedure used to analyze the polymer in previous studies is known to destroy most of the 1,6-linkages (12). The present studies thus demonstrate that all of the linkages found in the pentasaccharide are contained in a high molecular weight glycoprotein that is similar in carbohydrate composition to cell envelope heteroglycan.

It is important to note, however, that glycosyl transfer to endogenous acceptor apparently results only in the transfer of single mannosyl residues to nonreducing terminii and that synthesis de novo of the polymer does not occur. All attempts to demonstrate the involvement of lipid-intermediates in glycosyl transfer to either endogenous or exogenous acceptor have proven negative. This may indicate that synthesis of the saccharide chain of C. laurentii heteroglycan proceeds by stepwise transfer of glycosyl units from sugar nucleotides to the polysaccharide resembling glycoprotein biosynthesis in higher organisms (1, 2).

Note Added in Proof—Recent experiments indicate that disaccharides isolated after partial hydrolysis of endogenous [¹⁴C]-mannosyl product may contain some [¹⁴C]mannose at the reducing terminal. Apparently some newly transferred [¹⁴C]mannosyl residues can act as acceptors for subsequent mannosyl transfer.

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REFERENCES
