Biosynthesis of Oligogalactosyl Side Chains of the Cell Envelope Glycoprotein of Cryptococcus laurentii*

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MOHAN K. RAIZADA, HANS G. KLOEPFER,§ JOHN S. SCHUTZBACH,§ AND HELMUT ANKEL¶

From the Department of Biochemistry, The Medical College of Wisconsin, Milwaukee, Wisconsin 53223

SUMMARY

A particulate enzyme preparation from the fungus imperfectus Cryptococcus laurentii var. flavescens (NNRL Y-1401) catalyzes the transfer of galactosyl residues from UDP-[14C]galactose to an endogenous acceptor. The radioactive reaction product is particle-bound, but is solubilized by pronase treatment. The solubilized radioactive material migrates as a single peak on a column of Sepharose 4-B, indicating that the galactosyl residues are transferred to an endogenous polymeric acceptor of high molecular weight. After elution from Sepharose 4-B the material was found to contain 70% carbohydrate and 30% protein. The monosaccharides released after acid hydrolysis are mannose, galactose, and xylose, galactose being the only radioactive monosaccharide constituent. The radioactive reaction product is retained on DEAE-cellulose, from which over 80% of it is eluted with 0.5 m NaCl together with endogenous carbohydrate and protein. After treatment with NaOH-NaBH₄, the radioactive material no longer binds to DEAE-cellulose. Subsequent gel filtration on Sephadex G-25 results in a symmetrical radioactive peak of approximately 2000 molecular weight, suggesting liberation of a dodecasaccharide chain. Treatment with NaOH-NaB₃H₄ followed by complete acid hydrolysis allows isolation of tritium-labeled alanine and Cu-aminoacetic acid, indicating that carbohydrate side chains are linked through seryl and threonyl residues to protein. The major radioactive disaccharides isolated after partial acid hydrolysis are 6-O-α-[14C]galactosyl-0-[14C]galactose and 0-β-[14C]galactosyl-O-mannose. The data suggest addition of multiple galactosyl residues to an endogenous glycoprotein acceptor: the first galactosyl residue is linked to a mannosyl residue forming a β linkage; subsequently additional galactosyl residues are transferred sequentially forming linear chains containing 6-O-α-galactosyl-O-galactosyl linkages.

Cryptococcus laurentii is an encapsulated, yeast-like organism that is classified among the fungi imperfecti, and is closely related to the human pathogen Cryptococcus neoformans, the cause of cryptococcal meningitis. C. laurentii has a complex cell envelope containing a β-linked glucan, an acidic capsular polysaccharide composed of mannose, xylose, and glucuronic acid, and neutral glycoprotein containing mainly mannose, xylose, and galactosyl residues (1). Previous studies employing a particulate enzyme fraction isolated from cell-free extracts of this organism, have demonstrated transfer of mannose from GDP-mannose (1) and of xylose from UDP-xylose (2) to this cell envelope glycoprotein. This enzyme fraction was shown to contain at least four distinct mannosyl transferases (3) and two different xylosyl transferases (2). Using this enzyme fraction we have been able to synthesize de novo a branched pentaaccharide containing 1 xylose and 4 mannose residues (4). A corresponding pentaaccharide side chain is presumably bound to the cell envelope glycoprotein. The present report presents evidence that the same enzyme preparation catalyzes transfer of galactosyl residues from UDP-galactose to an endogenous acceptor that resembles the same cell envelope glycoprotein. Our data suggest that a dodecasaccharide side chain is synthesized: the first galactosyl residue is transferred to protein-bound mannosyl residues forming a β linkage; subsequently additional galactosyl residues are sequentially added to form linear chains containing 6-O-α-galactosyl-O-galactosyl linkages.

EXPERIMENTAL PROCEDURE

Materials—α-Gal-6-galactose was obtained by partial acid hydrolysis of stachyose or by acid reversion of galactose (5), α-Gal-4-galactose by partial acid hydrolysis of okra pod mucilage (6), α-Gal-3-galactose by partial acid hydrolysis of blood group B substance (7), and α-Gal-2-galactose from earthworms (8). The structures of these reference compounds were verified by cleavage with β-galactosidase and lead tetraacetate degradation (9). CDP-[14C]galactose and TDP-[14C]galactose, prepared from their corresponding glucosyl derivatives by incubation with UDP-galactose 4-epimerase from Escherichia coli, were kindly supplied by Dr. U. S. Maitra (10). A sample of ADP-[14C]galactose was a generous gift from the International Chemical and Nuclear Corporation. All other chemicals were obtained from commercial sources. A partially purified preparation of α-galactosidase was prepared from coffee beans (11). Using p-nitrophenyl-α-galacto-

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¶ Present address, Max Planck Institut fuer Molekulare Genetik, Berlin-Dahlem, Germany.
§ Present address, Department of Microbiology, The University of Alabama in Birmingham, Birmingham, Ala.
¶ Recipient of Career Development Award 1 K04 CM 50503 from the National Institute of General Medical Sciences, National Institutes of Health.
side as the substrate in 0.5 mM ammonium acetate buffer, pH 5.3, its specific activity at 25°C was 0.7 amole hydrolyzed per hour per mg of protein. Contaminating β-galactosidase, determined with the corresponding β-galactoside under identical conditions, was less than 2%.

**Chromatography and Electrophoresis—**Paper chromatography was carried out on Whatman No. 1 paper, either ascending or descending. The solvents used were: 1, 1-propanol-ethanol-acetate-water, 7:1:2; 2, 1-propanol-acetic acid-water, 6:1:2; 3, 2-propanol-1-butanol-water, 1:7:2; 4, 59% ethanol-1 mM ammonium acetate, pH 7.5; 5, ethyl acetate-pyridine-H₂O, 8:2:1; 6, phenol-H₂O-ammonium acetate, pH 4:1:1. Paper electrophoresis was carried out on Whatman No. 1 paper in 0.05 mM sodium borate, pH 9.2, at 30 volts per cm for 8 hours. Carbohydrates were detected with p-anisidine phthalate (12) or with silver nitrate-acetone followed by alcoholic sodium hydroxide spray (12).

**Enzyme Preparation—**Cryptococcus laurentii var. flaveavescens (NRRL Y-1401) was grown and harvested as described previously. Likewise the preparation of cell-free extracts and the isolation of the particulate enzyme fraction followed procedures previously outlined (1). The transferase with UDP-galactose as the substrate resided entirely in the middle layer of the 100,000 g particulate enzyme fraction. No attempts were made to solubilize the transferase activity. When stored at 0°C, full activity was retained for about 1 day; after 3 days at 0°C, the enzyme usually loses about 50% of its activity.

**Enzyme Assay—**Reaction mixtures contained UDP-[14C]galactose (0.05 μCi; 254 mCi per mmole), MnCl₂ (15 mM), Tris buffer, pH 7.0 (0.2 M), and enzyme. The mixture was made to 0.1 M sodium metaperiodate at pH 4.0 in a final volume of 0.1 ml. At different time intervals 20-μl aliquots were removed and added to 50 μl of 20% ethylene glycol in water. After thorough mixing the samples were applied on a double layer of Whatman DE81 anion exchange paper discs (2.7-cm diameter) and the discs were washed with 20 to 30 ml of deionized water using a Millipore filtration apparatus. Radioactive formate on the discs was measured directly in a liquid scintillation counter as described above.

**Results**

**Characteristics of the Galactosyl Transfer Reaction**

The 100,000 X g particulate middle layer fraction (middle layer) from C. laurentii catalyzes transfer of [14C]galactosyl residues from UDP-[14C]galactose to an endogenous acceptor. Incubation mixtures containing UDP-[14C]galactose, MnCl₂, and enzyme, after suitable times of incubation, were subjected to paper chromatography in Solvent 4, and the amount of polymer radioactive product at the chromatographic origin was determined.

Transfer of [14C]galactose to polymeric product proceeds linearly with time for at least 3 hours and is proportional to the enzyme concentration employed. The pH optimum of the reaction is close to 7. The galactosyl transfer reaction requires the presence of divalent cations, with maximal activity occurring at a concentration between 15 and 20 mM MnCl₂. Taking the reaction rate at 15 mM MnCl₂ as 100, other divalent cations tested at the same concentration yield the following activities: Fe²⁺, 13; Ca²⁺, 10; Mg²⁺, 2; Cu²⁺, Ni²⁺, Zn²⁺, less than 1. The apparent K₅, for UDP-galactose is 0.12 mM. The enzyme is quite specific for UDP-galactose. ADP-[14C]-galactose, CDP-[14C]galactose and TDP-[14C]galactose are not substrates for galactosyl transfer using the conditions of the standard assay. The addition of the following unlabeled sugar nucleotides at 1 mM concentration singly or in any possible combination does not appreciably increase or inhibit galactosyl transfer from UDP-[14C]galactose: UDP-glucose, UDP-galacturonate, UDP-xyllose, GDP-mannose.

**Properties of the Reaction Product**

As in the case of the reaction product obtained from GDP-[14C]mannose, the [14C]galactosyl product is particle-bound and...
insoluble in water but is solubilized with ethylene glycol. Digestion with pronase was found to be an easier method for the solubilization of the [\(^{14}\)C]galactosyl product and is described below. Reaction mixtures containing enzyme and reactants at concentrations as described for the assay, but scaled up 100-fold, were incubated for 24 hours. After centrifugation at 10,000 × g for 30 min, the sediment was washed 2 to 3 times with 3 ml of 0.5 M Tris buffer, pH 7.0. The insoluble material then was solubilized with sodium dodecyl sulfate at a final concentration of 4%. After removal of insoluble material by centrifugation at 10,000 × g for 10 min, 90% of the previously sedimentable radioactivity remained in the supernatant. Upon addition of 5 volumes of 95% ethanol, the radioactive material was quantitatively precipitated. The precipitate, isolated by centrifugation, was washed three times with 5 ml of water. The insoluble material then was suspended in 1 ml of 0.1 M Tris buffer, pH 8, and 5 mg of pronase were added. After incubation of 24 hours at 20°C another 5 mg of pronase were added and incubation was continued for another 24 hours. After centrifugation usually 80 to 90% of the label was found in the supernatant. When the solubilized material was applied to Sephadex G-200 or Sepharose 6B columns equilibrated with 0.5% ammonium acetate and eluted with the same solution, all of the radioactivity appeared in the void volume.

Column chromatography on Sepharose 4B in 0.5% ammonium acetate-1% sodium dodecyl sulfate resulted in a retarded peak in which label, endogenous carbohydrate, and 280-nm absorbing material migrated together (Fig. 1A). Differences in the ratios of carbohydrate and absorption in different fractions are most probably due to the low amounts of absorbing material present and corresponding large errors in the measured absorbance values. The radioactive material, when applied on a DEAE-cellulose column, was completely withheld by the column. Most of it (85%), however, was eluted with 0.5 M NaCl, with an additional small amount (15%) being eluted with 1 M NaCl (Fig. 2). The figure shows the elution profiles of endogenous carbohydrate and 280-nm absorbing material, which coincide with the label. The combined peak fractions contained 70% carbohydrate and 30% protein.

**Structural Analyses**

Complete Hydrolysis—Complete acid hydrolysis of the purified product followed by paper chromatography in Solvents 1 and 2 revealed the presence of mannose, galactose, and xylose, galac-

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**Fig. 1.** A, elution profile of pronase-solubilized [\(^{14}\)C]galactosyl product on a Sepharose 4B column. The [\(^{14}\)C]galactosyl product was treated with pronase and passed through a column of Sepharose 6B as described in the text. Radioactive material eluted from the column was concentrated under reduced pressure. An aliquot of this material was mixed with solubilized endogenous acceptor prepared by pronase treatment of the particulate enzyme preparation and Sepharose 6B gel filtration under identical conditions. The mixture containing 0.5% ammonium acetate and 1% sodium dodecyl sulfate then was applied on a Sepharose 4B column (27 × 1.5 cm) equilibrated and eluted with 0.5% ammonium acetate-1% sodium dodecyl sulfate. Carbohydrate was determined with phenolsulfuric acid, galactose being used as reference standard. B, NaOH-NaB\(_3\)H\(_4\)-treated [\(^{14}\)C]galactosyl product was applied on a DEAE-cellulose column as described in the text. The tritium-labeled material eluted from DEAE-cellulose with 1 M NaCl was passed through a column of Sephadex G-200. The tritium-labeled peak that appeared in the void volume was concentrated under reduced pressure and applied on the same Sepharose 4B column as in A. The column was equilibrated and eluted with 0.5% ammonium acetate. The figure shows the elution profile obtained. T-2000, peak elution volume of dextran T-2000 (Pharmacia, average molecular weight approximately 2 × 10\(^9\)); Gal, peak elution volume of standard galactose.

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**Fig. 2 (left).** Elution profile of pronase-solubilized [\(^{14}\)C]galactosyl product from a column of DEAE-cellulose. The radioactive material obtained in the void volume after passage through Sepharose 6B as described in the text was applied on a DEAE-cellulose column (16 × 1 cm) equilibrated at pH 8, and subsequently eluted with 0.5 mM potassium phosphate buffer, pH 7.5, 2-ml fractions being collected. Elution was continued in a stepwise fashion with increasing concentrations of NaCl as indicated in the figure. Carbohydrate was determined as explained in the legend of Fig. 1A.

**Fig. 3 (center).** Elution profile of pronase-solubilized [\(^{14}\)C]-galactosyl product after NaOH-NaB\(_3\)H\(_4\) treatment on a calibrated Sephadex G-25 column (28 × 1.5 cm). The column was equilibrated and eluted with 0.5% aqueous ammonium acetate and calibrated with authentic glucose, maltose and maltotriose.

**Fig. 4 (right).** Paper chromatographic separation of hydrolysis products of the high molecular weight material obtained after NaOH-NaB\(_3\)H\(_4\) treatment of [\(^{14}\)C]galactosyl product as described in Fig. 1A. Hydrolysis was carried out in 6 M HCl and 110°C as described in the text. After removal of HCl under reduced pressure, an aliquot of the hydrolysate was mixed with [\(^{14}\)C]-labeled authentic alanine and \(\alpha\)-aminobutyric acid and subjected to paper chromatography in Solvent 6. The chromatogram was then cut into narrow strips and \(^{14}\)C and \(^{3}H\) were determined in a liquid scintillation counter. 1, serine; 2, threonine; 3, alanine; 4, \(\alpha\)-aminobutyric acid.
of the ethylenediamine-solubilized reaction product always contained glucose and sometimes small amounts of arabinose (1). The glucose is presumably derived from glycogen or glucon present in the middle layer fraction, whereas the arabinose found previously appears to be an artifact of the hydrolysis. Both sugars are absent in hydrolysates of the pronase-solubilized product. Therefore, further analyses were carried out after solubilization with pronase.

Treatment with NaOH-NaBH₄—When purified radioactive reaction product was treated with a mixture of 0.1 mM NaOH and 0.4 mM NaBH₄ under conditions that are known to cleave glycosyl- seryl or glycosyl-threonyl linkages (20h, 18 hours, Ref. 16), it was no longer retained on DEAE-cellulose, suggesting that indeed such linkage(s) had been cleaved. Subsequent chromatography of the NaOH-NaBH₄-treated radioactive reaction product on a calibrated column of Sephadex G-25 revealed a symmetrical peak of radioactive material with an approximate molecular weight of 2000 (Fig. 3), suggesting that treatment with NaOH-NaBH₄ and liberated an oligosaccharide side chain with the size of a dodecasaccharide.

NaOH-NaBH₄ treatment was also carried out in the presence of 13 mM NaBH₄ (specific activity, 138 mCi per mmole) under otherwise identical conditions. Chromatography of the reaction product on DEAE-cellulose resulted in two fractions: nonabsorbed material containing both [³H] and [¹⁴C] labeled material containing only [³H], which subsequently was eluted with 1 M NaCl. Chromatography of the nonabsorbed material on Sephadex G-25 showed a tritium-containing peak that coincided with the [¹⁴C]-containing peak. The [³H]:[¹⁴C] ratio in this peak was calculated to be 5.8:1. Complete acid hydrolysis of the doubly labeled material, followed by paper chromatography in Solvent 3, revealed [¹⁴C]galactose and a tritium-labeled compound with the paper chromatographic mobility of mannitol, clearly separated from authentic galactitol, sorbitol, and xylitol.

The tritium-labeled product that was eluted from the DEAE-cellulose column with 1 M NaCl was further purified by gel filtration on Sephadex G-200. The tritium-containing material that eluted in the void volume was applied on Sepharose 4B. As seen in Fig. 18, a major peak containing tritium label with a mobility slightly slower than that of the intact pronase-solubilized product was eluted. The combined peak fractions were taken to dryness under reduced pressure and then hydrolyzed in 6 M HCl for 24 hours at 110°. After removal of HCl under reduced pressure, the hydrolysate was mixed with samples of authentic [¹⁴C]galactose, [¹⁴C]mannose, [¹⁴C]arabinose, [¹⁴C]glucosamine, [¹⁴C]glutamic acid, and subjected to paper chromatography in Solvents 6 and 7. The paper chromatograms then were cut into narrow strips and each strip was monitored directly for [¹⁴C] and [³H] in a liquid scintillation counter. As Fig. 4 illustrates for Solvent 6, tritium-labeled material moves identically to [¹⁴C] standards of α-amino[¹⁴C] butyric acid and [¹⁴C]alanine. This was confirmed by chromatography of another sample in Solvent 7. The data indicate that during NaOH-NaBH₄ treatment seryl and threonyl residues are converted to the corresponding dehydro amino acids as expected for β elimination, and that subsequent reduction of the double bonds by NaBH₄ results (17). We conclude from these data, that the pronase-solubilized radioactive reaction product is, indeed, glycoprotein in nature.

Periodate Treatment—Complete periodate oxidation of the purified [¹⁴C]oligosaccharide component liberated 11.2% of the total radioactivity as [¹⁴C]formate. In reference to the amount of [¹⁴C]formate liberated from control methyl[¹⁴C]galactopyrano- side (see “Experimental Procedure”), this value corresponds to 0.76 mole of [¹⁴C]formate per mole of [¹⁴C]galactosyl unit in the oligosaccharide. This value is consistent with a structure, in which either all of the newly transferred [¹⁴C]galactosyl units are terminal or are arranged in 1,6-linked chains.

Partial Acid Hydrolysis—Acetolysis of the solubilized product according to Stewart et al. (18) liberated most of the radioactivity as free galactose, suggesting that a major portion of the newly formed galactosyl linkages is 1,6. Partial acid hydrolysis was carried out in 5% sulfuric acid at 80° for 1 hour. The hydrolysate was cooled in ice and neutralized with solid barium hydroxide and removal of precipitated barium sulfate by centrifugation, the supernatant was subjected to ascending paper chromatography in Solvent 1 three consecutive times. The figure shows a radioautogram of the chromatogram, also the standards that were revealed by p-anisidine phthalate spray: left, galactose; right, α-Gal-6-galactose.

Fig. 5. A reaction mixture containing 7.3 moles of UDP-[¹³C]galactose (2 µCi), 20 µmoles of MnC₂O₄ and enzyme (36 mg of protein) in a total volume of 2.5 ml of 0.1 M Tris-HCl buffer, pH 7.0, was incubated for 18 hours at 20°. The [¹³C]galactosyl product was solubilized with pronase and purified by Sephadex G-200 column chromatography as described in the text. The radioactive material eluting with the void volume of the column was dried under reduced pressure and hydrolyzed in 1 ml 5% sulfuric acid at 80° for 1 hour. After neutralization with solid barium hydroxide and removal of precipitated barium sulfate by centrifugation, the supernatant was subjected to ascending paper chromatography in Solvent 1 three consecutive times. The figure shows a radioautogram of the chromatogram, also the standards that were revealed by p-anisidine phthalate spray: left, galactose; right, α-Gal-6-galactose.
TABLE I
Labeled oligosaccharides obtained after partial hydrololysis of reaction product

The radioactive bands corresponding to those shown in Fig. 5 were eluted and counted in a gas flow counter. Radioactivity eluted from the chromatographic origin was 0.29 μCi. Each individual saccharide was passed through a Bio-Gel P-2 column (82 × 1.5 cm) equilibrated and eluted with 0.5% aqueous ammonium acetate. Fractions of 1 ml were collected. The column was calibrated with standards of glucose, maltose, and higher maltotriose oligosaccharides. Their respective peak elution volumes were: glucose, 111 ml; maltose, 106 ml; maltotriose, 102 ml; maltotetraose, 88 ml; maltopentaose, 90 ml.

<table>
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<tr>
<th>Band</th>
<th>Radioactivity</th>
<th>Peak elution volume</th>
<th>Degree of polymerization</th>
<th>Structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0.45</td>
<td>111</td>
<td>1</td>
<td>Galactose</td>
</tr>
<tr>
<td>B</td>
<td>0.03</td>
<td>106</td>
<td>2</td>
<td>β-Gal-mannose</td>
</tr>
<tr>
<td>C</td>
<td>0.10</td>
<td>106</td>
<td>2</td>
<td>α-Gal-6-galactose</td>
</tr>
<tr>
<td>D</td>
<td>0.03</td>
<td>102</td>
<td>3</td>
<td>α-Gal-6-β-Gal-mannose</td>
</tr>
<tr>
<td>E</td>
<td>0.10</td>
<td>98</td>
<td>4</td>
<td>(α-Gal-6)-β-Gal-mannose</td>
</tr>
<tr>
<td>F</td>
<td>0.20</td>
<td>90</td>
<td>5</td>
<td>(α-Gal-6)-α-Gal-mannose</td>
</tr>
</tbody>
</table>

Analysis of Radioactive Oligosaccharides—Disaccharide B has the same mobility as enzymatically synthesized O-β-galactosyl-O-mannose upon paper chromatography in Solvents 1, 2, 3, and 5 and upon paper electrophoresis in barbitrate buffer. It is resistant to α-galactosidase treatment, but [14C]galactose is quantitatively liberated after treatment with β-galactosidase, as shown by chromatography in Solvent 2 (Fig. 6A). After NaBH₄ reduction of disaccharide B and complete acid hydrolysis, only [14C]galactose was released; no radioactive galactitol was formed.

When reduction was carried out in the presence of NaBH₄, [1H]mannitol was produced as established after complete acid hydrolysis and paper chromatography in Solvent 3 (Fig. 7A). These data as well as the enzymatic synthesis of a β-[14C]galactosyl-mannose disaccharide from UDP-[14C]galactose in the presence of C. laurentii middle layer enzyme and free mannose as exogenous acceptor, together with the identical electrophoretic and chromatographic mobilities in four solvents, suggest that disaccharide B, indeed, is O-β-[14C]galactosyl-O-mannose. The linkage of this disaccharide has yet to be determined.

Disaccharide C migrated as a single spot with the mobility of authentic α-Gal-6-galactose upon further paper chromatography in Solvents 1, 2, and 5 and upon paper electrophoresis in barbitrate buffer. The chromatographic mobility of α-Gal-6-galactose is clearly different from that of authentic α-Gal-4-galactose, α-Gal-3-galactose, and α-Gal-2-galactose. Complete acid hydrolysis quantitatively liberated galactose as the only radioactive material. Also treatment with α-galactosidase quantitatively released galactose; treatment with β-galactosidase was without effect (Fig. 6B). When an aliquot of Band C was subjected to reduction with NaBH₄ prior to complete acid hydrolysis and the reaction mixture was subsequently separated by paper chromatography in Solvent 3, two radioactive spots with the mobilities of authentic galactose and galactitol in a ratio of 1:1 were observed (Fig. 7B). These data demonstrate that Band 3 using descending paper chromatography in Solvent 1, the following &lactose values have been found: α-Gal-2-galactose, 0.54; α-Gal-3-galactose, 0.50; α-Gal-4-galactose, 0.39; α-Gal-6-galactose, 0.31.
C is a disaccharide containing 2 radioactive galactose units linked in α-1,6 linkage. Reduction of Band C also was carried out with NaBH₄ as described under "Experimental Procedure," but omitting subsequent addition of unlabeled NaBH₄. The reduced disaccharide then was isolated by column chromatography on Sephadex G-25. The ratio of ³H:¹⁴C in the fractions containing both labels was calculated to be 28.4:1.

Oligosaccharides isolated from Bands D, E, and F (trimer, tetramer, and pentamer) were all shown to be resistant to β-galactosidase treatment, but were partially degraded by α-galactosidase. As seen in Fig. 8A, Band D, when partially digested with α-galactosidase, showed liberation of [¹⁴C]galactose and a radioactive disaccharide with a mobility identical with that of Band B and to synthesized β-Gal-mannose. The paper chromatographic mobilities remained identical in Solvents 1, 2, 3, and 5. Elution of this disaccharide and subsequent treatment with β-galactosidase resulted in complete hydrolysis. Partial acid hydrolysis of Band D with 5.0% sulfuric acid at 80°C for 1 hour followed by neutralization with Ba(OH)₂ and chromatography in Solvent 1 showed, in addition to free galactose and unhydrolyzed material, a radioactive spot with the mobility of the reducing end also was revealed, when the intact trisaccharide was reduced with NaBH₄, then hydrolyzed and analyzed by column chromatography in Solvent 2. No radioactive galactitol was observed; all radioactivity migrated as free galactose.

Bands E and F, upon partial hydrolysis with α-galactosidase, both liberated free [¹⁴C]galactose and radioactive disaccharides with the chromatographic mobility of Band B. Again, these disaccharides were shown to have paper chromatographic mobilities identical to Band B in Solvents 1 and 2 and to contain both a [¹⁴C]galactosyl residue bound in β linkage to an unlabeled monosaccharide, presumably mannose. Intermediate breakdown products of Band E after α-galactosidase treatment were radioactive trisaccharide, and of Band F, radioactive tri- and tetrascarbohydrate, as judged from paper chromatographic mobilities of the hydrolysis products. Partial acid hydrolysis of Bands E and F performed as described for Band D liberated 6-O-α-[¹⁴C]galactosyl-0-[¹⁴C]galactose in both cases. Thus, Bands E and F most likely are (6-O-α-[¹⁴C]galactosyl)-0-β-[¹⁴C]galactosyl-mannose and (6-O-α-[¹⁴C]galactosyl)-0-β-[¹⁴C]galactosyl-0-mannose.

**DISCUSSION**

The data presented in this publication show the involvement of two galactosyltransferase reactions in the synthesis of an oligogalactosyl side chain of C. laurentii cell envelope glycoprotein: one that results in galactosyl transfer to terminal galactosyl residues forming 6-O-α-[¹⁴C]galactosyl-0-galactosyl linkages, the other yielding 0-β-galactosyl-0-mannosyl linkages to terminal mannosyl residues. Since the radioactive reaction product obtained contains both types of newly synthesized linkages, the apparent Michaelis constant and other kinetic parameters that were determined must represent those of the rate-limiting reaction.

The radioactive material released from the endogenous reaction product by NaOH-NaBH₄ treatment appears to be a decaascaccharide with mannitol at the reducing terminal. The results of periodate treatment suggest that either all [¹⁴C]galactosyl residues are terminal, or that they are arranged in linear 1,6-linked chains. Assuming that the complete oligosaccharide contains only 1 mannosyl unit at the reducing end, isolation of a [¹⁴C]galactosyl-mannose disaccharide from partial acid hydrolysates suggests that at least some of the oligogalactosyl chains are synthesized de novo. The conclusion that previously transferred [¹⁴C]galactosyl residues act as acceptors for subsequent attachment of additional [¹⁴C]galactosyl units is evidenced by the isolation of 6-O-α-[¹⁴C]galactosyl-0-[¹⁴C]galactose from partial hydrolysates of the reaction product and further supported by the analytical data for the isolated, tri-, tetra-, and pentascarbohydrides.

The reduced oligosaccharide chain liberated by NaOH-NaBH₄ treatment contained tritium and ¹⁴C in a ratio of 5.8:1. The 6-O-α-[¹⁴C]galactosyl-0-[¹⁴C]galactose disaccharide isolated from partial acid hydrolysates of the [¹⁴C]galactosyl product showed a ³H:¹⁴C ratio of 28.4:1 after reduction with NaBH₄, under comparable conditions. Assuming that both purified oligosaccharides were not contaminated by other unlabeled oligosaccharides of similar size and chromatographic behavior, the amounts of tritium incorporated correspond to the molar amounts of both oligosaccharides present. Thus, the quotient of the ³H:¹⁴C ratios of reduced decaascaccharide and reduced disaccharide, 5.8:28.4, should equal 2/X, in which X represents the total number of [¹⁴C]galactosyl residues per decaascaccharide chain. This calculation results in a value of 9.8, suggesting that the structure of the newly synthesized oligosaccharide chains is (6-O-α-[¹⁴C]galactosyl)-0-β-[¹⁴C]galactosyl-0-mannosyl-
which \( n \) equals at least 9 and probably 10. The known lability of glycosyl-serine and glycosyl-threonine linkages in the presence of NaOH-NaBH₄ suggests that in the original enzymatic [14C]galactosyl product, reducing terminal mannosyl units are linked through serine or threonine residues (or both) to the peptide portion of the glycoprotein. This conclusion is supported by the demonstration of tritium-labeled alanine and \( \alpha \)-aminobutyric acid in hydrolysates of NaOH-NaBH₄-treated [14C]galactosyl product.

Using the same particulate enzyme preparation as described in this publication, previous studies have shown that [14C]mannosyl residues are transferred from GDP-[14C]mannose to an endogenous acceptor resembling the acceptor for [14C]galactosyl residues (1). The mannosyl-mannosyl linkages that are formed are \( \alpha-1,2 \), \( \alpha-1,3 \), and \( \alpha-1,6 \) (1, 4). These transfer reactions were shown to be catalyzed by three distinct mannosyltransferases, specific for the formation of one type of bond (4).

Starting with exogenous mannose or \( \alpha \)-methylmannoside as acceptor, the three mannosyltransferases together with a xylosyltransferase present in the particulate enzyme preparation (2) mediate stepwise synthesis de novo of a branched heteropenta-oligosaccharide. Starting with exogenous mannose or \( \alpha \)-methylmannoside as acceptor, the three mannosyltransferases together with a xylosyltransferase present in the particulate enzyme preparation (2) mediate stepwise synthesis de novo of a branched heteropentasaccharide in the presence of GDP-mannose, UDP-xylose, or UDP-galactose and the divalent cations Mn²⁺ and Mg²⁺. The structure of this pentasaccharide is \( 2-O-\alpha \)-mannosyl-6-O-\( \alpha \)-mannosyl-3-O-\( \alpha \)-mannosyl-(2-O-\( \beta \)-xylosyl)-O-mannose (4). This pentasaccharide, however, when incubated in the presence of the same enzyme preparation, GDP-mannose, UDP-xylose, or UDP-galactose and a variety of metal ions, including Mn²⁺ and Mg²⁺, did not accept additional mannose, xylose, or galactosyl residues. We therefore conclude that it represents the structure of a completed side chain of the cell envelope glycoprotein, and that the galactosyl oligosaccharide is a separate side chain on the same glycoprotein. This interpretation is supported by the following experiments (19).

Pronase-solubilized reaction products obtained by either [14C]mannosyl or [14C]galactosyl transfer to endogenous acceptor, were both shown to bind to concanavalin-A, a plant lectin that binds terminal \( \alpha \)-mannosyl residues, but not terminal \( \alpha \)-galactosyl residues. After treatment with NaOH-NaBH₄, only the [14C]mannosyl-containing material retains its affinity for concanavalin-A; the [14C]galactosyl material no longer binds to the lectin. Whereas the [14C]galactosyl-material released after NaOH-NaBH₄ has the size of a dodecasaccharide, the major radioactive oligosaccharide released from the [14C]mannosyl product is a pentasaccharide containing the same linkages as those found in the branched pentasaccharide that can be synthesized de novo.

We have been unable to detect transfer of [14C]galactosyl residues from UDP-[14C]galactose to lipid-soluble material, although formation of a [14C]mannosyl lipid has been observed in the presence of GDP-[14C]mannose (1). This may indicate that synthesis of oligogalactosyl chains of \( C. laurentii \) cell envelope glycoprotein proceeds by stepwise transfer of galactosyl units from UDP-[14C]galactose directly to the growing chain. It is quite possible that mannosyl lipid is involved in the transfer of the first, protein-bound mannosyl. This possibility is suggested by recent findings of Babiczinski and Tanner, who have demonstrated that in \( S. cerevisiae \) only the mannosyl residue directly linked to protein is incorporated via its dolichol monophosphate derivative (20).

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Biosynthesis of Oligogalactosyl Side Chains of the Cell Envelope Glycoprotein of *Cryptococcus laurentii*
Mohan K. Raizada, Hans G. Kloepfer, John S. Schutzbach and Helmut Ankel


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