Mannosyl Transfer in *Cryptococcus laurentii* 

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**SUMMARY**

A particle-bound enzyme from the fungus imperfectus *Cryptococcus laurentii* var. flavescens (NRRL Y-1401) mediates transfer of mannose units from GDP-mannose to endogenous primer. The enzyme requires divalent cations for activity. The pH optimum is 7.5 and the apparent $K_m$ for GDP-mannose is 0.14 mm. A polysaccharide fraction that is associated with the enzyme was found to be similar to a polysaccharide fraction that can be isolated from intact cells or from cell wall preparations by ethylenediamine extraction and fractionation with Cetavlon. Both polysaccharide fractions contain mannose, galactose, arabinose, xylose, and glucose; both are soluble in the presence of Cetavlon. In contrast, an acidic polysaccharide produced by the organism contains mannose, glucuronic acid, and xylose and is Cetavlon-insoluble. The radioactive reaction product that is obtained from GDP-mannose-14C remains particle-bound, but is solubilized with ethylenediamine. The solubilized product is Cetavlon-soluble and nondialyzable. Complete hydrolysis releases mannose as the only D-monomer. Structural analyses of acetylation products of the enzymatic product reveal that mannosyl residues are newly linked to nonreducing ends of the enzyme-bound primer, resulting in α-1,2- and α-1,3-mannosyl-mannose bonds. Comparison of electron micrographs of whole cells or cell wall preparations before and after ethylenediamine extraction suggests that a polymer similar to the enzymatic product resides in the cell wall.

Fungi of the genus *Cryptococcus* are nonfermenting yeast-like organisms that are classified among the fungi imperfecti and multiply by budding (see, however, Reference 2). All species belonging to this genus are encapsulated (3). In addition to forming capsules, several species excrete extracellular polysaccharides (4). An acidic polysaccharide that is excreted by *Cryptococcus laurentii* contains mannose, 1 xylose, and glucuronic acid (5). UDP-glucose, GDP-mannose, and UDP-xylose, probably precursors of polysaccharide biosynthesis in this organism, have been isolated previously (6). A particulate enzyme that catalyzes transfer of xylosyl residues from UDP-xylose to partially dexylosylated extracellular polysaccharide has been described (7).

We have studied the role of GDP-mannose in polysaccharide biosynthesis of *C. laurentii*. We have investigated a particulate enzyme that transfers mannose units from this sugar nucleotide to an enzyme-bound polysaccharide acceptor. Analytical data show that the enzymatic reaction product is different from the acidic polysaccharide, but resembles a heteropolysaccharide that is associated with the cell wall.

In this paper some properties of the mannosyl transferase are described and analytical data of the enzymatic reaction product are compared to characteristics of isolated cell wall components.

**EXPERIMENTAL PROCEDURE**

**Materials**—All chemicals were obtained from commercial sources except the following: Man-2-mannose, 1 Man-2-Man-2-mannose, and Man-3-Man-2-Man-2-mannose were prepared from *Saccharomyces cerevisiae* mannan (Pierce Biochemicals) (8). The mannan was prepared and partially deoxyxylated extracellular polysaccharide has been described previously (6). A particulate enzyme that catalyzes transfer of xylosyl residues from UDP-xylose to partially deoxyxylated extracellular polysaccharide was prepared according to the method described by Abercrombie et al. (5). The L-arabinose dehydrogenase (EC 1.1.1.46) was prepared from *Pseudomonas saccharophila* (ATCC 15946) according to Doudoroff (12).

**Enzyme Preparation**—*C. laurentii* var. flavescens (NRRL Y-1401) was grown and harvested as described previously (18). The standard medium consisted of 0.1% urea, 0.1% KH$_2$PO$_4$.

1 The abbreviations used are: Man-2-mannose, 2-O-α-D-mannosyl-β-D-mannose; Man-2-Man-2-mannose, (2-O-α-D-mannosyl)-β-D-mannose; Man-3-mannose, 3-O-α-D-mannosyl-β-D-mannose; Man-6-mannose, 6-O-α-D-mannosyl-β-D-mannose; Man-2-arabinose, 2-O-α-D-mannosyl-β-arabinose.

1 All sugars are of the $D$ configuration unless otherwise noted in the text.
yield was 0.3 /&i.

Centrifugation and dissolved in 10 ml of 10% NaCl solution, a final concentration of 0.120/ The sediment was collected by the dialyzed material by centrifugation. To the supernatant water. Small amounts of insoluble matter were removed from was dissolved in 10 ml of water and dialyzed against 6 liters of the supernatant was collected. Ethylenediamine was evaporated in approximately 1 volume of the same buffer and was disrupted by ultrasonic treatment in a Branson Sonifier (model 125) for 7 min at an output of 11 amps.

The cell debris was removed by centrifugation at 12,000 × g for 15 min; the particulate fraction was then isolated by centrifugation at 105,000 × g for 50 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 matter separated into three distinct layers. Top, middle, and bottom layers were carefully isolated and resuspended in approximately 3 volumes of the same buffer. The transferase with GDP-mannose as the substrate resided entirely in the middle layer. No attempts to solubilize the enzyme have been undertaken. The transferase activity was unchanged for at least 4 weeks of storage at -20°. Repeated freezing and thawing were, however, avoided, since activity losses were often observed after such treatment.

Isolation of Cell Wall Polysaccharides from Whole Cells—Two 500-ml Erlenmeyer flasks each containing 100 ml of standard medium and 2% glucose (w/v) were inoculated from a starter flask and grown to midlog phase. They were then harvested by centrifugation under sterile conditions, washed once with standard medium, and resuspended in 100 ml of standard medium containing 50 µCi of glucose-14C (40 µCi per mmole). After 5 hours of growth on a shaker at 28°, 90% of the radioactivity had disappeared from the medium, while the cell population had doubled. The cells were then harvested by centrifugation and washed repeatedly with 1% NaCl solution until the washings were essentially free of radioactivity. Two more washings with distilled water (10 volumes) were carried out to remove sodium chloride. The packed cells were suspended in 20 ml of ethylene diamine and stirred at room temperature for 72 hours. The extracted cells were then removed by centrifugation and the supernatant was collected. Ethylenediamine was evaporated by distillation under reduced pressure and the resulting syrup was dissolved in 10 ml of water and dialyzed against 6 liters of water. Small amounts of insoluble matter were removed from the dialyzed material by centrifugation. To the supernatant Cetavlon (hexadeyltrimethylammonium bromide) was added to a final concentration of 0.12%. The sediment was collected by centrifugation and dissolved in 10 ml of 10% NaCl solution, whereafter 3 volumes of 95% ethanol were added. The sediment was collected by centrifugation, washed once with 70% ethanol, and dried. This fraction is referred to as "Cetavlon-insoluble polysaccharide from cells." Its yield was 6 µCi.

The supernatant that remained after removal of the Cetavlon precipitate was concentrated to 1 ml and then 3 volumes of ethanol were added. The resulting suspension was kept at 0° overnight; the precipitate was collected by centrifugation, washed once with 70% ethanol, and dissolved in 1 ml of water. This fraction is referred to as polysaccharide "in vitro." Its yield was 0.5 µCi.

Isolation of Cell Wall Polysaccharides from Isolated Cell Wall—Electron microscopy revealed that the "top layer" (see "Enzyme Preparation") represented rather uniform preparations of cell wall fragments (Fig. 10A). Therefore, the top layer isolates of 3 liters of C. laurentii culture (400 mg, dry weight) were subjected to extraction with ethylenediamine and fractionation with Cetavlon as described above. As seen in Fig. 10B, only part of the cell wall is solubilized by ethylenediamine treatment. The yield of Cetavlon-insoluble polysaccharide was 43 mg and that of Cetavlon-soluble polysaccharide was 3 mg.

Analytical Methods—Carbohydrate was determined with phenol-sulfuric acid (14), glucose with Glucostat (Worthington), galactose with Galactostat (Worthington), pentoses according to Roe and Rice (15), mannose by an adaptation of the enzymatic glucose determination described by Pfeiderer (16), and protein according to the method of Lowry et al. (17). Radioactivity was measured in a gas-flow counter (Nuclear-Chicago) or in a liquid scintillation counter (Packard) with Bray's solution (18) or tolune scintillation fluid (Spectrafloor, Amersham/ Searle). Paper chromatograms and electrophoretograms were analyzed for radioactivity with a Nuclear-Chicago Actigraph or by radioautography.

Chromatography and Electrophoresis—Paper chromatography was carried out on Whatman No. 1 paper, either ascending or descending. The solvents used were: 1, 85% ethanol-1 M ammonium acetate, pH 7.5-0.1 M disodium ethylenediaminetetraacetate, 70:30:1; 2, 1-propanol-ethyl acetate-water, 7:1:2; 3, ethyl acetate-pyridine-water—3α, 70:25:5, 3b, 70:25:5, 3c, 6:3:1:0 paper impregnated with KCl by dipping it into 3% KCl solution and drying at 110°; 4, isobutyric acid-1 M ammonium hydroxide, 5:3; 5, acetone-1-butanol-water, 7:2:1; 6, 1-butanol-pyridine-water, 10:3:7; 7, water-saturated phenol. Electrophoresis was carried out on Whatman No. 1 paper at 30 volts per cm for 8 hours in 0.04 M sodium borate, pH 9.2. Carbohydrates were detected with p-anisidine phthalate, silver nitrate-acetone followed by alcoholic sodium hydroxide spray (19) or periodate-benzidine (20).

Enzyme Assay—Reaction mixtures contained 0.1 µCi of GDP-[14C]-mannose (151 µCi per mmole), 0.8 µmole of MgCl2, 5 µmole of Tris-HCl buffer (pH 7.5), and enzyme (0.25 to 0.7 mg of protein) in a total volume of 0.05 ml. Incubations were carried out at 25° in sealed capillary tubes. The reactions were terminated by submerging the reaction vessels in boiling water for 3 min. The reaction mixtures were then subjected to descending chromatography in Solvent 1. Appropriate areas at the origin were cut out and counted in a gas-flow counter.

Reduction with NaBH4—Reductions were carried out by adding 20 µl of 0.5 M NaBH4 to a solution of radioactive oligosaccharide in 20 µl of water, leaving the reaction mixture at room temperature overnight, deionizing it with cation exchanger, and removing boric acid by repeated evaporation in the presence of methanol.

Acetylation—This was carried out essentially as described by Stewart, Mendershausen, and Ballou (21), but the time of acetylation was changed to 16 hours and the temperature to 35°. Under these conditions, optimal amounts of disaccharides appeared to be formed. After methanolysis the acetylation products were separated in Solvent 2.

Lead Tetraacetate Oxidation—Samples were evaporated to dryness and redissolved in 0.1 ml of glacial acetic acid. A solution of 5 mg of lead tetraacetate in 0.4 ml of glacial acetic acid was
amounts of label become associated with the 100,000 X g pellet enzyme (Fig. 1). The amount of label incorporated into the 

The standard assay was used as described under "Experimental 

0.1 ml samples were withdrawn and subjected to centrifugation at 0° and 120,000 X g for 50 min. The radioactivity in the superna-

tant was determined in a gas flow counter. 

Fig. 2 (right). Mannosyl-14C transfer as a function of time. The standard assay was used as described under "Experimental Procedure." 

then added and the reaction mixture was kept at 298° for 10 min. The reaction was stopped by the addition of 0.2 ml of a 1% solution of oxalic acid in glacial acetic acid. The resulting precipitate was removed by centrifugation; the supernatant was deionized with mixed bed ion exchanger after dilution with 1.5 ml of water (11).

**RESULTS**

**Observations upon High Speed Centrifugation**—Increasing amounts of label become associated with the 100,000 X g pellet when GDP-mannose-14C is incubated with the middle layer enzyme (Fig. 1). The amount of label incorporated into the sediment levels off after approximately 2 hours, when about 50% of the substrate has been utilized (Experiment A). When half of the amount of middle layer enzyme is used (Experiment B), the rate of incorporation and the maximal amount incorporated are approximately half of that observed in Experiment A. Enzyme previously incubated at 25° for 2 hours does not show measurable loss in activity. These data show that C. laurentii contains a particulate enzyme that converts radioactivity from GDP-mannose-14C into a sedimentable product and suggest that this product might be a particle-bound polymer. They also suggest that termination of the reaction after 2 hours might be due to limiting primer. The following studies are carried out with the chromatographic assay as described under "Experimental Procedure."

**Effects of Incubation Time, Enzyme Concentration, and Substrate Concentration**—The incorporation of mannose from GDP-mannose-14C into a chromatographically immobile product is linear for at least 30 min under the conditions of the assay (Fig. 2). The activity varies linearly with the enzyme concentration (Fig. 3). In Fig. 4 the dependence of the reaction rate on the concentration of GDP-mannose is plotted according to Lineweaver and Burk (22). The apparent K_m for GDP-mannose calculated from these data is 0.14 mM.

**Effect of pH**—The dependence of the rate of mannose incorporation from the pH of the incubation mixture is illustrated in

**Fig. 5 (left).** Mannosyl-14C transfer as a function of pH. The standard assay was used as described under "Experimental Procedure." The following buffers at final concentrations of 0.1 M were used: pH 6.0 to 7.5, sodium phosphate; pH 7.5 to 8.75, Tris-HCl; pH 9.8, sodium carbonate. 

**Fig. 6 (right).** Mannosyl-14C transfer as a function of metal ion concentration. The standard assay was used as described under "Experimental Procedure," except that the metal ion concentration was varied as indicated in the figure.

**Metal Ion Requirement**—Divalent cations are essential for the transfer reaction; taking the reaction rate at 20 mM Mn^{++} as 100, other divalent cations tested at the same concentration give the following activities: Mg^{++}, 35; Co^{++}, 16; Zn^{++}, 16; Ca^{++}, 15; Ni^{++}, 4; Cu^{++}, <1. The corresponding chlorides were tested throughout. As can be seen in Fig. 6, both Mn^{++} and Mg^{++} exhibit maximal activation at a concentration of about 15 mM; higher concentrations inhibit progressively. No difference in the apparent K_m for GDP-mannose was noted when measured in the presence of either Mn^{++} or Mg^{++}, indicating that the different maximal activities in the presence of either Mn^{++} or Mg^{++} cannot be attributed to a change in the substrate K_m.

**Substrate Specificity**—When in the standard assay GDP-mannose-14C is replaced by equal concentrations of mannose-1-C-P or mannose-14C, no labeled product is formed; addition to the standard assay of unlabeled GDP-gluos or UDP-gluos in amounts equal to that of GDP-mannose-14C does not decrease the rate of incorporation of label into the product. 

**Primer Requirement**—The enzyme is active in the absence of
added primer. No increase of the rate of incorporation of mannose-\(^{14}C\) into polymer or of the maximal amount that is incorporated upon prolonged incubation (5 hours) is observed when the following cell fractions or polysaccharides are added to standard reaction mixtures at concentrations up to 10 mg per ml (dry weight): top layer, boiled middle layer, boiled bottom layer, extracellular polysaccharide, partially dexylosylated extracellular polysaccharide. Cetavlon-insoluble polysaccharide, Cetavlon-soluble polysaccharide, bakers' yeast mannan. Since boiled middle layer enzyme does not show any effects on rate as well as on the maximal amount of mannose-\(^{14}C\) that is incorporated, the enzyme-bound acceptor appears to be active as a primer only in the "native" enzyme-acceptor complex.

Effects of Other Sugar Nucleotides—The following unlabeled sugar nucleotides, when added at 1 mm concentration to standard reaction mixtures, do not result in appreciable increases in the rate of mannose incorporation or in the maximal amount of label that is incorporated after 4 hours of incubation: UDP-glucose, TDP-glucose, UTP-glucuronic acid, UDP-xyllose, UDP-galactose, UDP-L-arabinose, or any combination thereof. A 100-fold excess of the mentioned sugar nucleotides over the GDP-mannose that is incorporated, the enzyme-bound acceptor appears to be active as a primer only in the "native" enzyme-acceptor complex.

"Lipid Intermediate"—Radioautograms of reaction mixtures that were separated in either Solvent 1 or 4 reveal labeled material close to the solvent front ("lipid"). When Mn\(^{2+}\) is omitted from the reaction mixtures, neither polymer nor lipid is formed. The relative amounts of label in the lipid vary between 20 and 80% of the amount that is incorporated into the polymer when different enzyme preparations are used. The lipid is water-insoluble, but can be extracted from reaction mixtures with 1-butanol-pyridinium acetate (a mixture of 2 volumes of 1-butanol and 0.5 volume of 6 M acetic acid, adjusted to pH 4.2 with pyridine (23)) or a 2:1 ratio of extractant and reaction mixture was used. It is soluble in chloroform-methanol (1:2) after extraction, but cannot be extracted from reaction mixtures by this solvent. These properties are similar to those reported for the lipid intermediate in bacterial cell wall biosynthesis (23). Hydrolysis of the lipid in 1 N HCl for 3 hours at 100° releases all of the radioactivity as free mannose as shown by paper chromatography (Solvent 2). Hydrolysis for 15 min at 100° liberates the following amounts of radioactive mannose: 1 N HCl, 88%; 0.1 N HCl, 19%; 0.01 N HCl, 16%; 0.005 N NaOH, <1%. Treatment with NaOH results in a water-soluble product that remains at the chromatographic origin in Solvent 2, whereas unhydrolyzed lipid has an \(R_{manno}\) value of 1.6. The observed resistance to acid hydrolysis is in contrast to known properties of lipid intermediates in a variety of polysaccharide-synthesizing systems (23-25). Standard reaction mixtures containing isolated lipid (2 μCi) instead of GDP-mannose fail to reveal transfer of label to polymer, even when the lipid is emulsified by sonic oscillation (26) or by the addition of detergents. When formation of lipid and polymer is studied with time, the kinetics of lipid formation is different from that described for truly established lipid intermediates: lipid formation does not precede mannosyl incorporation into polymer and a steady state level of the lipid is not observed (Fig. 7). Attempts to chase the label from lipid into polymer by addition of unlabeled GDP-mannose to reaction mixtures that are previously incubated with labeled GDP-mannose do not result in a decrease of label in the lipid material. This indicates that labeled mannose is not chased from lipid to polymer and thus does not support the concept of an intermediate that turns over rapidly (Fig. 7). Although we had speculated that a mannosyl-lipid is an intermediate in the transfer reaction, the material detected does not appear to serve this function.

**Reaction Product**—The chromatographically immobile reaction product is not eluted from the paper with water, 1 N NaCl, 1 N acetic acid, 1 N ammonia, ethanol, butanol, or chloroform-methanol (1:1). It is, however, quantitatively extracted with ethylenediamine. For structural analysis reaction mixtures scaled up by a factor of 25 to 50 were subjected to ethylenediamine extraction as described under "Experimental Procedure." After removal of the solvent by distillation under reduced pressure followed by dialysis against water, 50 to 90% of the radioactivity is nondialyzable and water-soluble, whereas water-insoluble material that is removed by centrifugation contains only little radioactivity. When Cetavlon at a final concentration of 0.12% is added to the aqueous solution containing the radioactive product, a precipitate is formed; 90% of the radioactivity remains in the supernatant after removal of the precipitate by centrifugation. Increasing the Cetavlon concentration to 0.3% does not result in further precipitate formation. Upon addition of 3 volumes of 95% ethanol to the supernatant the radioactive material is quantitatively precipitated. Since a major contaminant of the radioactive reaction product is glycogen, further purification of the ethanol precipitate was sought by treatment with α-amylase (from hog pancreas, EC 3.2.1.1). To an aqueous solution of 50 mg of the ethanol precipitate containing 0.15 μCi of radioactive material in 3 ml of water, 50 units of α-amylase were added and the reaction mixture was kept at 25° overnight. To the solution 3 volumes of ethanol were added and the resulting precipitate was washed three times with 20 ml of 70% ethanol. Amylase treatment was repeated three times until paper chromatographic examination revealed no further liberation of glycogen breakdown products (Solvent 2). No radioactivity was released into the soluble fraction by this treatment. As shown in Table I, α-amylase did not remove all of the
negligible amounts of free mannose are detected upon scanning of the chromatograms through a strip scanner, as illustrated. Monas saccharophila standards were eluted and counted in a gas flow counter. The ratio for 60% of the radioactivity accounted for and the analytical details are given in Table I. The individual sugars were carried out after eluting representative areas of a chromatogram that was obtained by chromatography of an identical hydrolysate (Solvent 3a). The ratios of the sugars were calculated assuming that all monosaccharides had the same specific activity per carbon atom. Numbers in parentheses represent the corresponding monosaccharide area after determination with the phenolsulfuric acid method (14), with the appropriate authentic monosaccharides as standards. Although these determinations were not carried out for the minor monosaccharide components, the similarity of the ratios as obtained by the two methods supports the assumption that the specific activities of the sugars in the polysaccharides in vivo are in fact quite similar.

Some radioactive material (between 20 and 30% in different preparations) was not hydrolyzed under the conditions used and remained at the chromatographic origin. Further hydrolysis with 2 N HCl for 6 hours at 100°C did not release any more of the above monosaccharides but several radioactive bands that comigrated with ninhydrin-positive material (Solvent 2). No material that migrated like glucuronic acid or glucuronic acid lactone was observed. This material was not analyzed further, but presumably represents protein.

The composition was determined after 3 hours of hydrolysis in 1 N HCl at 100°C, chromatography in Solvent 3a, and elution of representative areas of the chromatogram.

### Table I

**Composition of Polysaccharide Fractions Isolated from Cryptococcus laurentii**

The composition was determined after 3 hours of hydrolysis in 1 N HCl at 100°C, chromatography in Solvent 3a, and elution of representative areas of the chromatogram.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Molar ratios</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Arabinose</td>
</tr>
<tr>
<td>Enzymatic product</td>
<td>0.2</td>
</tr>
<tr>
<td>Cetavlon-soluble polysaccharide from cell wall</td>
<td>0.1</td>
</tr>
<tr>
<td>Polysaccharide in vivo a, b</td>
<td>0.1</td>
</tr>
<tr>
<td>Cetavlon-insoluble polysaccharide from cells c, d, e</td>
<td>0</td>
</tr>
</tbody>
</table>

a Determined according to Roe and Rice (15). An aliquot of each arabinose eluate was subjected to oxidation with Pseudomonas saccharophila L-arabinose dehydrogenase in the presence of DPN and resulted in the formation of the expected amount of DPNH.
b Determined according to Roe and Rice (15). An aliquot of each xylose eluate was incubated with 17 units of glucose oxidase (EC 1.1.3.4) and 200 units of peroxidase (EC 1.11.1.7.) at room temperature and pH 7 for 4 hours. Paper chromatography (Solvent 2) revealed complete oxidation of both samples.
c Determined with hexokinase (EC 2.7.1.1), ATP, pyruvate kinase (EC 2.7.1.40), phosphoenolpyruvate, lactate dehydrogenase (EC 1.1.1.27), and DPNH (16).
d Determined with glucose oxidase (Gluecoptst, Worthington).
e Determined with galactose oxidase (Galactoestat, Worthington).
f Radioactive areas with mobilities identical with authentic standards were eluted and counted in a gas flow counter. The radioactive monosaccharides isolated after hydrolysis accounted for 60% of the total radioactivity in the case of the polysaccharide glucose-containing polymer. A sample of the purified product (0.02 μCi) was hydrolyzed for 3 hours at 100°C in 1 N HCl. Paper chromatography (Solvents 2, 3a, 7) followed by p-anisidine phthalate spray revealed the following monosaccharides: mannos, glucose, galactose, arabinose, and xylose. Radioautography showed a single radioactive spot that comigrated with unlabeled mannose. Quantitative determinations of the individual sugars were carried out after eluting representative areas of a chromatogram that was obtained by chromatography of an identical hydrolysate (Solvent 3a). The ratios of the sugars found and the analytical details are given in Table I. The δ configuration of the isolated mannose is shown by its reactivity with hexokinase and ATP, that of galactose by its reactivity with lactose oxidase, and that of glucose and xylose (6) by their reactivity with hesokinase and ATP, that of galactose by its reactivity with galactose oxidase, and that of glucose and xylose (6) by their reactivity with glucose oxidase, and the δ configuration of the isolated arabinose was shown by its reactivity with L-arabinose dehydrogenase from Pseudomonas saccharophila.

When 0.01 μCi of the reaction product is incubated for 24 hours at pH 4.5 and 25°C with emulsin (1 mg) which contains α-mannosidase (EC 3.2.1.24; 8, 27), approximately 50% of the radioactivity is released as free mannose, shown after paper chromatography in Solvent 2 (Fig. 8). When the unreacted material is re-eluted and again treated under identical conditions, no further liberation of mannose is observed. When polysaccharide in vivo (0.01 μCi, see below) is treated identically, only negligible amounts of free mannose-14C are detected upon scanning of the chromatograms through a strip scanner, as illustrated in Fig. 8. When the chromatographic origin and the mannose area were excised and the radioactivity on both pieces of paper was determined with a liquid scintillation counter with toluene seintillation fluid, the following results were obtained: origin, 8000 cpm; mannose area, 100 cpm. Since mannose residues account for 20% of the radioactivity of the polysaccharide preparation in vivo used in this experiment, the amount of free mannose detected after emulsion treatment is 10% of the total amount of this sugar originally present.

It appears from this that in the polymer in vivo only a small fraction of the mannose units is terminal and available for cleavage with α-mannosidase, whereas approximately 50% of the enzymatically transferred mannose units are located at nonreducing terminals of the reaction product and are α-linked. The remaining part might have a different type of linkage or might be protected from mannosidase cleavage through steric hindrance by neighboring glycosyl units. Our structural studies which do not result in α-mannosidase-resistant disaccharides appear to favor the latter interpretation (see below).

### Comparison of Reaction Product with Cell Wall Polysaccharides

A polysaccharide with a composition comparable to that of the enzymatic reaction product has thus far not been described in C. laurentii. We therefore attempted to compare the product with various polysaccharide fractions that were obtained from intact cells or from isolated cell wall preparations. In Figs. 9 and 10 electron micrographs of whole cells and of isolated cell wall preparations (top layer) before and after ethylenediamine extrac-
the Cetavlon-soluble material, in addition to glycogen, contained considerable amounts of a glucan that could not be removed by treatment.

The reaction mixture was then dialyzed overnight against 4 liters of water. The dialyzed material was hydrolyzed anisidine phthalate spray did not indicate any decrease of the presence of dark-staining granules in the intact cell and within the cell wall preparation. These granules are composed of glycogen. They appear to remain inside the cells when whole cells are extracted, but mostly disappear upon extraction of the cell wall preparation.) Further fractionation of the cell wall was carried out with the ethylenediamine extract of intact cells. For this, cells grown on radioactive glucose as described under "Experimental Procedure" were extracted with ethylenediamine, and the extract, after removal of the ethylenediamine, was separated into a Cetavlon-insoluble and a Cetavlon-soluble fraction. The compositions of complete acid hydrolysates of both fractions obtained after chromatographic separation (Solvent 3a) and elution of the radioactive monosaccharides are given in Table I. The radioactive monosaccharides comigrated with authentic standards upon further paper chromatography in Solvents 2 and 7. It is evident that hydrolysates of the Cetavlon-soluble material that is extracted from cells or cell wall have a composition very similar to that of the enzymatic product. Note the low contents of free glucose in the polysaccharide obtained from intact cells as compared to that in the cell wall isolate. At the present time, we are not sure whether glucose is not in fact part of the cell wall heteropolysaccharide. The varying amounts of glucose found in what appears to be identical polysaccharide fractions tend to support the conclusion that at least part of this originates from a glucan that contaminates our neutral heteropolysaccharide fractions and is resistant to glycogen-degrading enzymes. It should be pointed out that the ratio of Cetavlon-soluble to Cetavlon-insoluble heteropolysaccharide in the cell is in the order of 1:15 to 20.

Acetolysis—Reaction product (0.3 μCi) and polysaccharide in vivo (0.1 μCi) were subjected to acetolysis in acetic acid, acetic acid anhydride, sulfuric acid (10:10:1) for 16 hours at 35°. After methanolysis, the reaction products were separated by ascending paper chromatography (Solvent 2). In Fig. 11 radioactive tracings of both chromatograms are represented. Under the conditions used with both polymers a major portion of the mannosyl residues is liberated as free mannose. In both cases practically no oligosaccharide was found that migrates like Man-2-Man-2-mannose. Whereas little radioactivity is associated with material that migrates like or slower than Man-3-Man-2-Man-2-mannose in the case of the polysaccharide in vivo, a considerable portion of the radioactivity in the acetolysate obtained from the polysaccharide in vivo is found close to or at the chromatographic origin. This difference might be due to the fact that in the polymer in vivo mannose is the only radioactive sugar associated with nonreducing terminal linkages, whereas in the polymer in vivo glycosyl residues throughout the whole molecule are radioactive. The disaccharide areas from both acetolyses were eluted. Both eluates migrate as single spots of identical mobility upon descending chromatography in Solvents 2 and 6. Authentic Man-2-Man-2-mannose and Man-3-mannose do not separate in these solvents and migrate identically with the above disaccharides, whereas authentic Man-6-mannose migrates more slowly in both solvents. Paper electrophoresis in borate buffer, however, resolves both radioactive disaccharides into two components with mobilities identical with authentic Man-2-mannose (I) and Man-3-mannose (II) (Fig. 12). The disaccharides I and II in vivo were present in a ratio of 2:3, and the disaccharides I and II in vitro in a ratio of 4:1. Although not too apparent from the figure, complete separation was obtained as judged after radioautography. Disaccharides I and II in vitro and in vivo were incubated with 1 mg of emulsin α-amylase or amyloglucosidase (from Aspergillus niger, EC 3.2.1.3 and EC 3.2.1.11) treatment, further analyses were carried out with the ethylenediamine extract of intact cells.

A sample (8 mg) of the Cetavlon-soluble polysaccharide from cell wall was incubated with 1 mg of amyloglucosidase (Sigma) in 1 ml of 0.05 m sodium acetate buffer, pH 4.5, for 5 hours at room temperature. The reaction mixture was then dialyzed overnight against 4 liters of water. The dialyzed material was hydrolyzed for 3 hours at 100° in 1 N HCl and subjected to chromatography (Solvent 3a). Visual inspection of the chromatogram after p-anisidine phthalate spray did not indicate any decrease of the amount of glucose present as compared to a control hydrolysate obtained from the polysaccharide without prior amyloglucosidase treatment.

Fig. 8. Treatment of the enzymatic reaction product (II) and of polysaccharide in vivo (III) with α-mannosidase (emulsin, Sigma). Samples (0.01 μCi) were incubated for 24 hours at pH 4.5 and 25° in the presence of 1 mg of enzyme. One milligram of emulsin cleaves 12 μmoles of 4-β-D-mannosyl-D-mannose per hour under these conditions; 4-β-D-mannosyl-D-mannose is not a substrate as shown after incubation of 0.1 mg with 1 mg of emulsin for 24 hours at pH 4.5 and 25°, followed by paper chromatography in Solvent 2. The figures show radioactive strip scans obtained after ascending paper chromatography of the reaction mixtures in Solvent 2. A control reaction containing enzymatic reaction product and boiled enzyme is shown in I. The compositions of complete acid hydrolysates of both fractions tend to support the conclusion that at least part of this originates from a glucan that contaminates our neutral heteropolysaccharide fractions and is resistant to glycogen-degrading enzymes. It should be pointed out that the ratio of Cetavlon-soluble to Cetavlon-insoluble heteropolysaccharide in the cell is in the order of 1:15 to 20.

Acetolysis—Reaction product (0.3 μCi) and polysaccharide in vivo (0.1 μCi) were subjected to acetolysis in acetic acid, acetic acid anhydride, sulfuric acid (10:10:1) for 16 hours at 35°. After methanolysis, the reaction products were separated by ascending paper chromatography (Solvent 2). In Fig. 11 radioactive tracings of both chromatograms are represented. Under the conditions used with both polymers a major portion of the mannosyl residues is liberated as free mannose. In both cases practically no oligosaccharide was found that migrates like Man-2-Man-2-mannose. Whereas little radioactivity is associated with material that migrates like or slower than Man-3-Man-2-Man-2-mannose in the case of the polysaccharide in vivo, a considerable portion of the radioactivity in the acetolysate obtained from the polysaccharide in vivo is found close to or at the chromatographic origin. This difference might be due to the fact that in the polymer in vivo mannose is the only radioactive sugar associated with nonreducing terminal linkages, whereas in the polymer in vivo glycosyl residues throughout the whole molecule are radioactive. The disaccharide areas from both acetolyses were eluted. Both eluates migrate as single spots of identical mobility upon descending chromatography in Solvents 2 and 6. Authentic Man-2-Man-2-mannose and Man-3-mannose do not separate in these solvents and migrate identically with the above disaccharides, whereas authentic Man-6-mannose migrates more slowly in both solvents. Paper electrophoresis in borate buffer, however, resolves both radioactive disaccharides into two components with mobilities identical with authentic Man-2-mannose (I) and Man-3-mannose (II) (Fig. 12). The disaccharides I and II in vivo were present in a ratio of 2:3, and the disaccharides I and II in vitro in a ratio of 4:1. Although not too apparent from the figure, complete separation was obtained as judged after radioautography. Disaccharides I and II in vitro and in vivo were incubated with 1 mg of emulsin α-amylase or amyloglucosidase (from Aspergillus niger, EC 3.2.1.3 and EC 3.2.1.11) treatment, further analyses were carried out with the ethylenediamine extract of intact cells.

A sample (8 mg) of the Cetavlon-soluble polysaccharide from cell wall was incubated with 1 mg of amyloglucosidase (Sigma) in 1 ml of 0.05 m sodium acetate buffer, pH 4.5, for 5 hours at room temperature. The reaction mixture was then dialyzed overnight against 4 liters of water. The dialyzed material was hydrolyzed for 3 hours at 100° in 1 N HCl and subjected to chromatography (Solvent 3a). Visual inspection of the chromatogram after p-anisidine phthalate spray did not indicate any decrease of the amount of glucose present as compared to a control hydrolysate obtained from the polysaccharide without prior amyloglucosidase treatment.
sidase at pH 4.5 and 25°C for 24 hours. This treatment quantitatively liberated mannose-3H as the only radioactive product, shown after paper chromatography in Solvent 2. These findings indicate that all four radioactive disaccharides are α-linked and that the disaccharides in vivo are 1,2- and 1,3-linked manno-

bioses. No further structural studies of both disaccharides in vivo have been carried out. When disaccharides I and II in vitro were reduced with NaBH₄, followed by hydrolysis in 1 N HCl for 1 hour at 100°C and paper chromatography (Solvent 5), all of the radioactivity was found in the mannose area; no radioac-
FIG. 11. Separation of acetolysis products of the enzymatic product (top) and of the polysaccharide in vivo (bottom) after chromatography in Solvent 2. The acetolysis conditions were those described under “Experimental Procedure.” (MAN), 2-O-α-D-mannosyl-α-D-mannose; (MAN)₂, 2-O-α-D-mannosyl-α-D-mannose; (MAN)₃, 3-O-α-D-mannosyl-2-O-α-D-mannosyl-α-D-mannose.

FIG. 12. Separation of radioactive disaccharides that were isolated after acetolysis (Fig. 10). Shown is the distribution of radioactivity after paper electrophoresis in 0.04 M sodium borate buffer, pH 9.2, carried out as described under “Experimental Procedure.” Top, disaccharides I and II in vitro; bottom, disaccharides I and II in vivo. α-1,2(MAN)₂, 2-O-α-D-mannosyl-α-D-mannose; α-1,3(MAN)₂, 2-O-α-D-mannosyl-α-D-mannose.

tive mannitol could be detected. When the reduction was carried out with NaBH₄ (approximately 1 mCi per μmole), chromatography of hydrolyzed samples in Solvent 3e (that separates mannitol from dulcitol and sorbitol) and Solvent 3b (that separates dulcitol from mannitol and sorbitol) showed 80% of the t-labeled material to migrate like mannitol. These data suggest that both disaccharides in vitro are also mannobioses containing a ¹⁴C-labeled mannosyl unit attached to a nonradioactive mannose. The major disaccharide component in vitro, disaccharide I, was subjected to short time lead tetraacetate degradation (10 min, 28°). The resulting radioactive product showed electrophoretic mobility identical with the untreated control. This fact suggests that no degradation has occurred. Whereas 1,3-, 1,4-, and 1,6-linked mannobioses are rapidly oxidized with lead tetraacetate, resistance to short time lead tetraacetate degradation is consistent with a 1,2-linkage (11). To confirm this linkage, the following experiment was carried out: disaccharide I in vitro (7400 cpm) was mixed with authentic Man-2-mannose (21 mg), dissolved in 2 ml of water, and reduced with NaBH₄ (10 mg) overnight at room temperature. After removal of excess NaBH₄ and boric acid by treatment with cation exchanger and repeated evaporation in the presence of methanol, the resulting mannosyl-mannitol was crystallized from ethanol-methanol (28). The specific radioactivity of the dried material was determined and recrystallization was repeated two more times. The observed specific activities were as follows: first crystals, 310; second, 370; third, 390; second mother liquor, 350; third mother liquor, 350 cpm per mg. These data confirm a mannosyl-¹⁴C,1,2-mannose-¹⁴C structure for the

**Table II**

<table>
<thead>
<tr>
<th>Oligosaccharide</th>
<th>$R_{Man}$</th>
<th>Fraction no. of peak tube</th>
<th>$DP_r$</th>
<th>Ratio of mannose to galactose</th>
<th>Presumed composition&lt;br&gt;&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>B in vivo</td>
<td>0.67</td>
<td>53</td>
<td>3</td>
<td>2:1.0</td>
<td>Man-Gal</td>
</tr>
<tr>
<td>B in vitro</td>
<td>0.70</td>
<td>53.5</td>
<td>3</td>
<td>2:1</td>
<td>Man-Gal</td>
</tr>
<tr>
<td>C in vivo</td>
<td>0.28</td>
<td>50</td>
<td>4-5</td>
<td>2:0.28</td>
<td>Man₂-Gal₂</td>
</tr>
<tr>
<td>C in vitro</td>
<td>0.28</td>
<td>50</td>
<td>4-5</td>
<td>2:3</td>
<td>Man₂-Gal₂</td>
</tr>
<tr>
<td>D in vivo</td>
<td>0.15</td>
<td>48</td>
<td>5-6</td>
<td>1:0.95</td>
<td>Man₁-Gal₃</td>
</tr>
<tr>
<td>D in vitro</td>
<td>0.14</td>
<td>48</td>
<td>5-6</td>
<td>1:1</td>
<td>Man₁-Gal₃</td>
</tr>
<tr>
<td>Maltotriose</td>
<td>59</td>
<td>2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Maltotetraose</td>
<td>54</td>
<td>3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Maltopentaose</td>
<td>49</td>
<td>5</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Paper chromatographic mobility in Solvent 2 relative to Man-2-Man-2-mannose.

<sup>b</sup> Each isolated oligosaccharide was subjected to gel filtration through a calibrated Sephadex G-15 column (2 × 27 cm). The column was eluted with water, 1 ml fractions being collected. Each fraction was dried down on a planchet and the radioactivity was determined in a gas flow counter. Authentic maltooligosaccharides were assayed with phenol-sulfuric acid (14).

<sup>c</sup> Approximate DP values for each oligosaccharide were estimated by comparison of the respective elution profiles obtained upon Sephadex filtration with those of the authentic maltooligosaccharides listed. DP, number of monomers per oligomer.

<sup>d</sup> Each oligosaccharide was hydrolyzed in 1 N HCl for 3 hours at 100° followed by paper chromatography in Solvent 2. In the case of the oligosaccharides in vivo, radioactive monosaccharides were located on the chromatograms by radioautography. The radioactive areas were excised and counted in toluene scintillation fluid in a liquid scintillation counter. The ratios listed are based on the actual counts that were measured (see Table I). In the case of the oligosaccharides in vitro, mannose was the only radioactive monosaccharide that was revealed after radioautography. The ratios listed are only approximations, since they were estimated from the intensities of the spots that were revealed upon spraying of the chromatograms with p-anisidine phthalate. The small amounts of material at hand precluded more exact determinations.

<sup>e</sup> Relative component residues in oligosaccharides.
that originated from the enzymatic product cochromatographed with endogenous oligosaccharides that were revealed upon p-anisidine obtained from the polysaccharide in V&O showed elution through a calibrated Sephadex G-15 column. The oligosaccharide profiles identical with those of the oligosaccharides obtained from the radioactive bands originating from the enzymatic product were each hydrolyzed with 1 N HCl for 3 hours and subjected to chromatography in Solvent 2. Mono- saccharide areas on the chromatograms containing radioactivity were cut out and counted in a scintillation counter in toluene scintillation fluid. The monosaccharide ratios as listed in Table II were calculated assuming equal specific activities for the respective monosaccharides (this appears justified from the data given in Table I). All three oligosaccharides obtained from the enzymatic product upon complete acid hydrolysis and chromatography in Solvent 2 and p-anisidine phthalate spray revealed the presence of mannose and galactose in each oligosaccharide. As seen in Table II, the monosaccharide compositions of the oligosaccharides in vivo resemble those estimated for the corresponding oligosaccharides of the enzymatic product. Although no detailed characterization of these oligosaccharides was carried out, the data suggest that the enzymatic product is quite similar to a polysaccharide component that is associated with the Cetavlon-soluble fraction of cell extracts. It also appears that the newly transferred radioac-

Fig. 13. Separation of acetylation products obtained after elution of radioactive material that migrated more slowly than 2-O-α-D-mannosyl-β-mannose after ascending chromatography in Solvent 2 (see Fig. 11). This material was subjected to descending chromatography in Solvent 2 for 170 hours. Top, enzymatic product; bottom, polysaccharide in vivo. Also shown are tracings of spots that were revealed with p-anisidine phthalate spray of a portion of the chromatogram containing the material derived from the enzymatic product. (MAN)α, (2-O-α-D-mannosyl)β-D-mannose; (MAN)α, 3-O-α-D-mannosyl-(2-O-α-D-mannosyl)-α-D-mannosyl units are in fact linked to a major polysaccharide component of the particulate enzyme fraction.

DISCUSSION

Intact cells or cell wall preparations of C. laurentii contain at least two heteropolysaccharide components that are released upon ethylenediamine extraction: a acidic component that is precipitable with Cetavlon and is composed of mannose, gluco-

DP represents the number of monomers per oligomer.
glycogen-cleaving enzymes, accompanies the heteropolysaccharide fractions obtained from broken cells. Bacon et al. (29) have presented data that indicate the presence of an α-1,3-linked glucan in the cell wall of two Cryptococcus species. Our data (30) show that the insoluble material remaining after ethylendiamine extraction of cell wall preparations is a glucan containing β-1,3- and β-1,6-linkages. Such materials or breakdown products thereof might conceivably account for the discrepancy in the amounts of glucose found in the various Cetavlon-soluble fractions. Arabinosyl and glucosyl units might be associated with the linkage region of the polymer in the native cell wall. Undoubtedly ethylendiamine causes the hydrolysis of some alkali-labile bonds that render this heteropolysaccharide water-soluble. Since glycopeptide linkages have been found in the cell wall of S. cerevisiae (31), it is possible that such linkages play a role in the binding of this heteropolysaccharide to the native cell wall of C. laurentii. The presence of amino acids in hydrolysis products of the Cetavlon-soluble fractions of the reaction product and the cell wall heteropolysaccharide has been noted. The possibility that the carbohydrate in both fractions is bound to protein is presently under investigation. We assume that the middle layer obtained after ultracentrifugation of cell-free extracts of C. laurentii contains partially disintegrated cell membranes that are associated with cell wall fragments. The composition of the Cetavlon-soluble polysaccharide fraction that is obtained after ethylendiamine extraction of middle layer enzyme is, in fact, almost identical with that obtained from isolated cell wall or from intact cells with the same isolation procedure.

As GDP-mannose has been previously shown in extracts of C. laurentii cells (6), it is not surprising that this sugar nucleotide acts as mannose donor in the biosynthesis of cell wall heteropolysaccharide. (The role of GDP-mannose in mannan synthesis in bakers' yeast (32) and Micrococcus lysodeikticus (24, 26) is well documented.) It is, however, surprising that mannose is transferred only to the neutral heteropolymer and not to the acidic one. Also, the rate of mannose transfer is not stimulated by a number of sugar nucleotides that conceivably should act as precursors of the other monosaccharide components in both polymers, nor is the maximal amount of mannose that can be transferred enhanced by such additions. These data indicate that under the conditions used polymer synthesis de novo does not occur. They also suggest that mannose units must be transferred to the acidic heteropolymer by a different mechanism. This is supported by the fact that xylosyl transfer to acidic extracellular polysaccharide is dependent on the addition of a primer that is obtained from the acidic polysaccharide (7). In contrast, mannose transfer occurs to enzyme-bound acceptor and is not stimulated by the addition of polysaccharide fractions obtained from the cells.7

The involvement of lipid-linked intermediates in the biosynthesis of cell wall homopolysaccharides in bacteria (23–26, 34) and in bakers' yeast (35) has been described. The observation in C. laurentii of the formation of a mannosyl-lipid concomitant with mannosyl transfer to polymer suggested to us the involvement of a lipid intermediate in cryptococcal polymer formation as well. The data reported in this communication provide some good evidence against the thesis that at least a major part of the mannosyl-lipid found is an intermediate in the transfer reaction studied here. It is possible that it is involved in synthesis de novo of mannos-containing heteropolymers of the cell wall, a process that we have not as yet been able to demonstrate. Further work on structure and possible function of this mannosyl-lipid is necessary and is being carried out.

Results of the analyses of the disaccharide obtained after acetylation of the radioactive reaction product show that at least two, and possibly three, different mannosyl linkages are formed. The complete lack of radioactivity in the reducing monosaccharide units of the disaccharides isolated from the enzymatic product indicates transfer of only one mannosyl unit and excludes the generation of new mannosyl acceptor sites after the addition of one mannos has occurred. The results of the α-mannosidase treatment also indicate that about 50% of the newly transferred mannosyl units are at nonreducing termini and are α-linked. The remaining 50% appear to be α-mannosidase-resistant. However, all of the isolated disaccharides were cleaved by α-mannosidase. This could have two possible reasons: either most of the α-mannosidase-resistant linkages are cleaved during acetylation, or 50% of the newly synthesized linkages are not accessible to the α-mannosidase in the intact polymer because of steric reasons, but are cleaved in the isolated disaccharide pieces. A similar phenomenon is described for the action of a bacterial mannosidase on bakers' yeast mannan (9). Whereas this enzyme cleaves only the α-1,2- and α-1,3-linked sidechains of the intact polymer and does not attack the α-1,6-linked backbone structure, α-1,6-linked mannose oligosaccharides up to hexamer are good substrates for the enzyme.

It is clear that the findings reported here do not provide an explanation for the biosynthetic mechanism by which cell wall heteropolysaccharide of C. laurentii is formed de novo. They describe a reaction in which mannose units are transferred to nonreducing ends of a previously existing heteropolymer. A similar mannosyl transferase has been found in M. lysodeikticus which also transfers only a small number of mannosyl units to nonreducing termini of a previously formed enzyme-bound mannan acceptor, although, in the latter system, a lipid intermediate has been clearly shown (24, 26).

A possible mechanism for cryptococcal polymer formation might be the stepwise synthesis of a linear heteropolysaccharide backbone to which in a final reaction mannosyl branches become attached. A growing oligosaccharide-lipid intermediate might function in the formation of the major portion of the polymer, whereas mannosyl branches are transferred directly from GDP-mannose by the reaction described in this communication.

Acknowledgments—We wish to thank Dr. C. E. Ballou for supplying the Arthrobacter sp. used in the preparation of Man-β-mannose. The skillful technical assistance by Miss Mary Aumann is gratefully acknowledged.

REFERENCES


7 We have subsequently found that mannos, Man-2-mannose, and Man-2-Man-2-mannose can act as exogenous acceptors in this system (33).
Mannosyl Transfer in *Cryptococcus laurentii*
Helmut Ankel, Else Ankel, John S. Schutzbach and John C. Garancis


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