Studies on the Biosynthesis of *Hansenula holstii* Mannans from Guanosine Diphosphate Mannose*

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ABSTRACT: A particulate enzyme fraction from *Hansenula holstii* catalyzes a Mn^{2+}-dependent transfer of [^{14}C]mannose from guanosine diphosphate [^{14}C]mannose to particle-bound acceptors. Approximately 50% of the particle-bound ^{14}C product is solubilized with hot Tris buffer (pH 8). The remaining particle-bound product is solubilized by Pronase digestion.

Both fractions are precipitable with Fehling's reagent, are excluded from Sephadex G-50, and yield mainly [^{14}C]mannose upon strong acid hydrolysis. The Tris-solubilized ^{14}C product, after Pronase digestion, is separated into two major ^{14}C fractions on DEAE-cellulose chromatography, as is also the Pronase-solubilized ^{14}C product. The first DEAE-cellulose fractions, eluted at low ionic strength, are characteristic of relatively neutral mannans. [^{14}C]Oligosaccharides of mannose through the tetrasaccharide are produced by acetolysis and these (the di- and tetrasaccharide) were shown to contain some [^{14}C]mannose in all of the hexose units. The second DEAE-cellulose fractions, eluted at higher ionic strength, contain phosphate and release [^{14}C]-mannose and [^{14}C]mannosylmannose upon mild acid hydrolysis (0.01 M HCl at 100°). No distinctive acetolysis products other than mannose and the disaccharide are produced. These overall properties are considered as characteristic properties of phosphoglycoproteins and phosphorylated mannans.

The major components of the yeast cell wall are mannan, glucan, and protein which are present in the form of macromolecular complexes. It is becoming increasingly apparent that phosphate, as phosphodiester, is an integral part of this complex and may be the linkage group between the constituent components (McLellan and Lampen, 1968; Sentandreu and Northcote, 1968; Stewart and Ballou, 1968). The phosphorus content of mannans isolated from several strains of yeast varies widely, as do the types of mannosidic linkages (Stewart and Ballou, 1968). Biosynthetic studies on *Saccharomyces carlsbergensis* mannan by Behrens and Cabib (1968) demonstrated the transfer of mannose from GDP-mannose to mannan acceptors. Evidence for a lipid intermediate in the biosynthetic pathway has been presented by Tanner (1969). The source of the phosphate remains unclear.

In an effort to further elucidate the structure and biosynthetic pathway of these macromolecules, we have initiated such studies with the yeast *Hansenula holstii*. In addition to having a cell wall mannan with a relatively high phosphorus content, this species excretes a highly phosphorylated mannan (Jeanes *et al.*, 1961; Slodki, 1962) and may therefore be of advantage in investigating the source of the phosphate. A particulate enzyme fraction has been obtained from *H. holstii* which catalyzes the transfer of both [^{14}C]mannose and [^{32}P]-phosphate from [β-^{32}P]GDP-[^{14}C]mannose to endogenous acceptors. We report here our studies on the properties and characterization of the [^{14}C]mannose transfer reaction and products. A preliminary account of this work and our work on the characterization of the ^{32}P product has been given (Kozak *et al.*, 1969; Bretthauer *et al.*, 1969).

Materials and Methods

Materials. GDP-[^{14}C]mannose was obtained from New England Nuclear or prepared enzymatically as described by Preiss and Greenberg (1967). [^{14}C]Mannose-1-P was obtained from GDP-[^{14}C]mannose by enzymatic hydrolysis with a purified yeast nucleotide pyrophosphatase (R. K. Haroz and R. K. Bretthauer, 1969, unpublished). The hydrolysis products were separated and purified by paper chromatography with solvents A and B.[1] [^{14}C]Mannose-6-P was prepared with crystalline yeast hexokinase and the product was isolated by chromatography in solvent B. Other unlabeled nucleotides and biochemicals were obtained from Sigma and Calbiochem. Bakers yeast and *H. holstii* mannans were prepared by alkaline extraction as described by Cifonelli and Smith (1955). Oligosaccharides (mannobiose through mannotetraose) used for markers on paper chromatography were prepared by acetolysis and Sephadex gel filtration as described by Lee and Ballou (1965) or by Stewart *et al.* (1968). Exocellular phosphomannan of *H. holstii* was isolated and acid hydrolyzed as previously described (Kozak and Bretthauer, 1968). The α-mannosidase was prepared from Jack bean meal (Li, 1967). *Escherichia coli* alkaline phosphatase was purchased from Worthington and Pronase from Calbiochem. *H. holstii* NRRL-Y 2448 was obtained from the Northern Regional Research Laboratories in Peoria, Ill.

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[1] The following solvent systems were used (in v/v): A, 95% ethanol–1 M ammonium acetate (pH 7.5) (7:3); B, 95% ethanol–1 M ammonium acetate (pH 5.0) (7:3); C, 1-butanol–pyridine–water (6:4:3); D, ethyl acetate–pyridine–water (5:3:2); E, 1-butanol–ethanol–water (5:1:4); F, methyl ethyl ketone–acetic acid–water-saturated boric acid (9:1:1).

TABLE I: Incorporation of Radioactivity from GDP-[^{14}C]-Mannose into Particulate Acceptors.[a]

Fraction	Radioactivity, cpm
Butanol–pyridine acetate soluble	92,000
Tris buffer soluble	182,000
Remaining particle bound	200,000

[a] Each incubation mixture of 10 contained the following components in 0.20 ml: 0.5 mg of particulate fraction protein; 25 mM imidazole acetate buffer (pH 6.5); 10 mM $MnCl_2$; 10 μM GDP-[^{14}C]mannose (1.5×10^5 cpm). After incubation for 60 min at 30°, the contents of the 10 tubes were pooled and added to 2 volumes of 6 M pyridine acetate (pH 4.2) in butanol (1:4, v/v). After shaking, the aqueous suspension was reextracted two more times with pyridine acetate–butanol. The combined butanol extracts were back-extracted three times with 2 ml of H_2O and then an aliquot was counted in the liquid scintillation counter. The remaining aqueous suspension after butanol extraction was centrifuged and the pellet was washed twice with 2 ml of H_2O by centrifugation. The washed pellet was suspended in 3 ml of 0.1 M Tris buffer (pH 8.1) and heated 30 min at 100°. After cooling and centrifuging, the pellet was washed twice with 1.5 ml of 0.2 M NH_4HCO_3. Aliquots of the combined supernatant solutions and the suspended pellet were chromatographed in solvent A for 12 hr and the radioactivity remaining at the origin was determined in the liquid scintillation counter.

Preparation of Particulate Fraction. Cells were grown as described previously by Anderson *et al.* (1960) with 2% glucose in a New Brunswick gyrotory shaker for 24 hr at which time the viscosity of the medium had increased considerably. The cells were harvested by centrifugation at 19,000*g* for 10 min and washed with 2 volumes of 1% KCl to remove the adherent polysaccharide. The cells were washed with 0.1 volume of 0.025 M imidazole acetate buffer (pH 6.5) containing 0.001 M mercaptoethanol and then suspended in 0.1 volume of the same buffer. The cells were ruptured by passing twice through a French pressure cell at 7000 psi. The broken cells were centrifuged at 1000*g* for 5 min to remove unbroken cells and cell wall debris. The 1000*g* supernatant solution was then centrifuged at 75,000*g* for 45 min. The pellet was washed by resuspending it in buffer and recentrifuging at 75,000*g* for 45 min. This procedure was repeated three times. The particulate fraction was free of whole cells as judged by microscopic examination and by lack of growth on nutrient agar plates. The pellet was suspended in the same buffer and stored in liquid nitrogen. Activity was maintained under these conditions for several months.

Assay Methods. A standard incubation mixture contained 25 mM imidazole acetate buffer (pH 6.5), 10 mM $MnCl_2$, 0.2–0.5 mg of particulate fraction protein, and the desired amount of nucleotide sugar (0.9–2.8 mμmole, specific activity of 1 μCi/μmole unless otherwise specified) in a total volume of 0.1 ml. Incubations were conducted at 30° and the reaction was terminated by heat denaturation for 2 min in a boiling-water bath. The amount of incorporation of radioisotope into the particulate fraction was measured by streaking the entire incubation mixture on paper chromatograms and irrigating with solvent A for 12 hr. The origin was cut out, immersed in scintillation fluid (4.0 g of 2,5-diphenyloxazole and 50 mg of 1,4-bis[2-(5-phenyloxazolyl)]benzene made up to 1 l. with toluene), and counted in a Packard Tri-Carb scintillation spectrometer (assay A).

Pronase digestions were carried out at 30° in 0.1 M Tris buffer (pH 8.0) and 0.01 M $CaCl_2$ with 200 μg of Pronase/ml. Alkaline phosphatase incubations contained either 1.0 M Tris buffer (pH 8.0) or 1.0 M NH_4HCO_3, and 20–50 μg/ml of enzyme. These were incubated at 30° for 20–30 hr. Hydrolysis with α-mannosidase was carried out in 0.1 M sodium citrate buffer (pH 4.5) for 5 hr at 30°.

Chromatography. Unless otherwise noted all paper chromatography was carried out on Schleicher and Schuell No. 589 Green Ribbon C paper in the descending direction. Paper electrophoresis was conducted with acetic acid washed Whatman No. 3MM paper and 0.2 M sodium borate buffer (pH 8.8) at 30 V/cm. Monosaccharides, oligosaccharides, and sugar alcohols were detected on paper with periodate–benzidine (Gordon *et al.*, 1956) and organic phosphate by the method of Isherwood and Barrett (1967). Isotopes on paper were detected with the Packard radiochromatogram scanner and by liquid scintillation counting.

Sephadex chromatography of ^{14}C products was carried out on 1 × 100 cm columns of Sephadex G-50 which were equilibrated with 0.2 M NH_4CO_3. The flow rate was 25 ml/hr. DEAE-cellulose chromatography was carried out on 1 × 7 cm columns previously equilibrated with 0.01 M NH_4CO_3. A linear gradient of 100 ml each of 0.01 M NH_4HCO_3 and 1.0 M NH_4HCO_3 was used to elute the ^{14}C products at a flow rate of 25 ml/hr. Fractions of 3 ml were collected.

Acetolysis and Sodium Borohydride Reduction of ^{14}C Products. Acetolysis was carried out essentially as described by Stewart *et al.* (1968), using a mixture of acetic anhydride, acetic acid, and sulfuric acid in a ratio of 10:10:1 (v/v). After 13 hr at 40°, excess pyridine was added to the solution, the mixture was evaporated to near dryness, and the acetates were extracted with chloroform. Deacetylation was carried out with sodium methoxide in methanol and solid carbon dioxide was added to neutralize the base. After evaporation, the mixture was applied directly to the chromatograph paper and developed with solvent C. The recovery of radioactivity was from 50 to 70%.

Reduction of the oligosaccharides was carried out by adding 60–70 μmoles of sodium borohydride to the 0.3-ml aqueous sample and allowing to stand at room temperature for at least 10 hr. After addition of 0.2 ml of acetone, Dowex 50 (H^+) resin was added, centrifuged out, and washed three times with 0.5 ml of methanol–H_2O (1:1). This solution was concentrated to dryness three times from methanol and the residue was dissolved in 1 M HCl. Hydrolysis was carried out in sealed tubes at 100° for 4 hr. After evaporation of the HCl in a desiccator over NaOH pellets, the residue was dissolved in H_2O and spotted on paper for chromatography in solvent F. Recovery of radioactivity in the mannose and mannitol areas of the chromatogram was about 50%.

Analytical Procedures. Protein was determined by the method of Lowry *et al.* (1951) using bovine serum albumin

TABLE II: Substrate Specificity of [^{14}C]Mannose Transfer Reaction.[a]

Substrate	Radioactivity, cpm
Expt 1	
GDP-[^{14}C]mannose	7200
[^{14}C]Mannose-1-P	0
[^{14}C]Mannose-6-P	0
Expt 2	
GDP-[^{14}C]mannose	204
GDP-[^{14}C]mannose + UDP-mannose	192
GDP-[^{14}C]mannose + IDP-mannose	110
GDP-[^{14}C]mannose + GTP	182

[a] In expt 1 standard incubation mixtures contained 2 mμmoles of either GDP-[^{14}C]mannose (2.5 μCi/μmole), [^{14}C]mannose-1-P (12.5 μCi/μmole), or [^{14}C]mannose-6-P (0.2 μCi/μmole). Incorporation of radioisotope was measured by assay A after a 20-min incubation. In expt 2, standard incubation mixtures contained 5 mμmoles of GDP-[^{14}C]-mannose (2.5 μCi/μmole), and where indicated, 50 mμmoles of either UDP-mannose, IDP-mannose, or GTP. Incorporation of radioisotope was measured by assay A after a 2-min incubation.

as standard, phosphate by the method of Ames and Dubin (1960), and carbohydrate by the phenol–H_2SO_4 method (Dubois *et al.*, 1956). Trimethylsilyl derivatives of carbohydrates from acid hydrolysates were prepared for gas–liquid partition chromatography as described by Sweeley *et al.* (1963). Precipitation of mannan with Fehling's reagent was carried out as described by Agranati *et al.* (1966).

Results

Mannosyl Transfer into the Particulate Fraction. The incorporation of [^{14}C]mannose from GDP-[^{14}C]mannose into endogenous acceptors is catalyzed by the particulate fraction. In Table I are shown results in which 6% of the added [^{14}C]-mannose is extracted, after incubation, by butanol–pyridinium acetate and about 50% of the remaining particle-bound radioactivity which remains at the origin after chromatography in solvent A is solubilized by heating in Tris buffer (pH 8). Additional experiments on the characterization of the Tris-soluble material will be presented later.

Kinetics of Mannose Incorporation. The amount of radioactivity incorporated from GDP-[^{14}C]mannose as determined with assay A in a 30-min incubation is proportional to the particulate fraction concentration of up to 500 μg of protein/0.1 ml of standard incubation mixture. The transfer reaction is proportional to time up to 50 min, and with prolonged times of incubation, or with high concentrations of particulate enzyme, up to 80% of the [^{14}C]mannose of the added GDP-[^{14}C]mannose is incorporated into product with assay A. The initial rate (2-min incubation) of incorporation into particulate-bound material was dependent on substrate concentration. The estimated K_m for GDP-mannose was 8.5 μM and V_{max} was 11×10^2 μμmole/min per mg of protein.

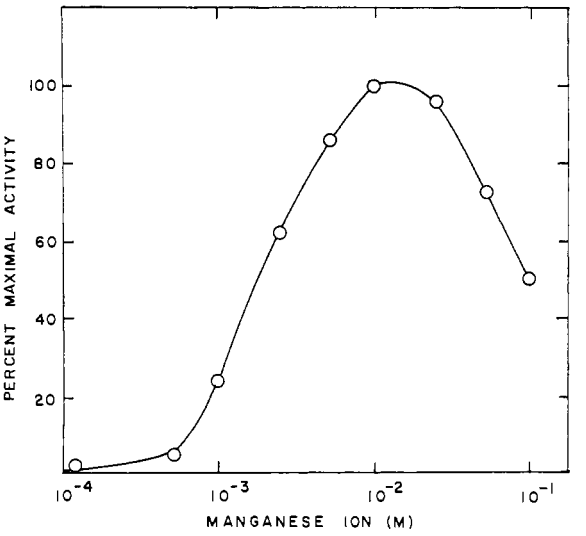


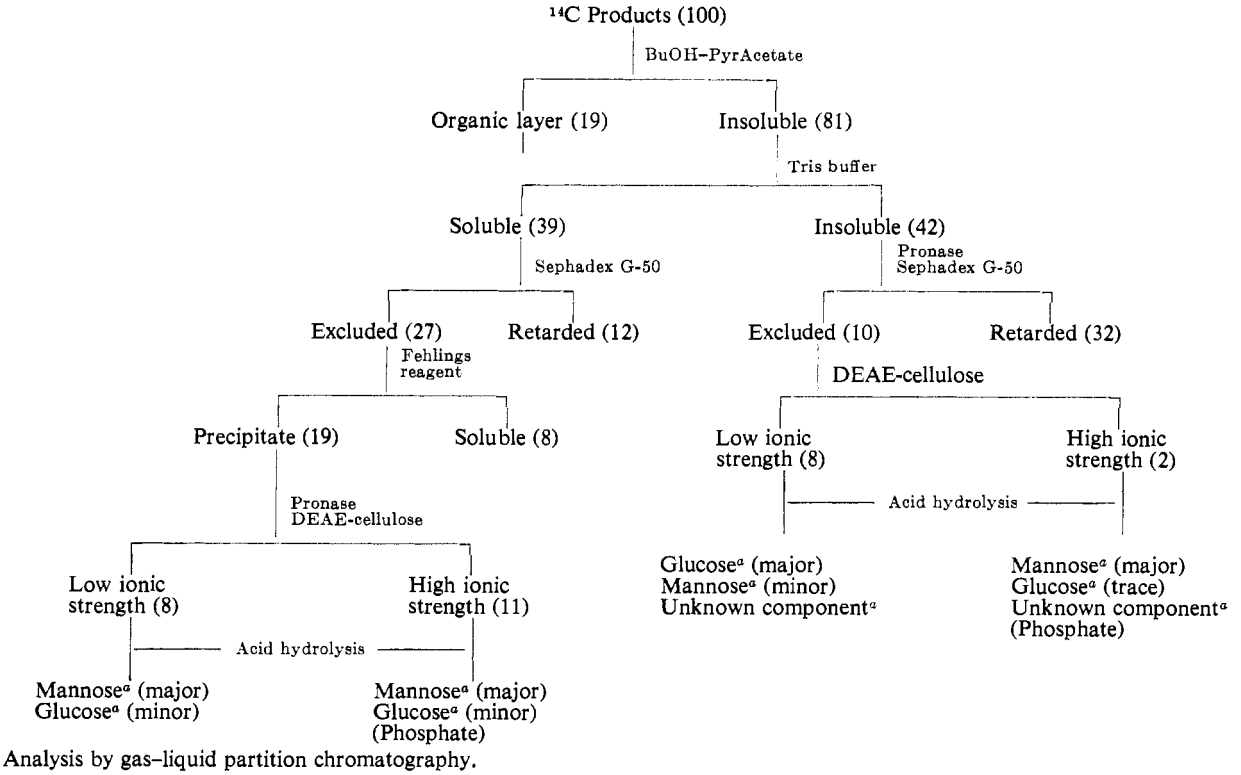
FIGURE 1: Effect of Mn^{2+} concentration on activity. The standard incubation mixtures contained 0.26 mg of particulate fraction protein and 1.8 mμmole of labeled GDP-mannose. Incorporation of [^{14}C]mannose was measured by assay A.

Metal Ion Requirement. The divalent cation requirement was very similar to that reported by Behrens and Cabib (1968) for cell wall mannan biosynthesis in. *S. carlsbergensis* with a protoplast membrane fraction. For [^{14}C]mannose incorporation, the order of effectiveness among the divalent cations tested at 10 mM concentration was $Mn^{2+} > Co^{2+} > Fe^{2+} > Mg^{2+} > Ca^{2+} > Zn^{2+} >$ none. Manganese ion was at least 2.5 times more effective than Co^{2+}. The optimal Mn^{2+} concentration was 10 mM (Figure 1).

Acceptor Requirements. Various fractions were prepared in order to test for acceptor activity. The following compounds were found to be negative; phosphorylated and dephosphorylated (with alkaline phosphatase) autohydrolysis products of *H. holstii* phosphomannan; *H. holstii* and *S. fragilis* mannans and their acetolysis products; protoplast membranes of *H. holstii* prepared by incubation with the intestinal juice of *Helix pomatia* (Kozak and Bretthauer, 1968); the particulate fraction carrying the transferase activity which was either heat denatured, hydrolyzed in 0.01 N HCl for 1 hr, hydrolyzed in 0.01 N HCl for 1 hr and then treated with alkaline phosphatase, or heat denatured and alkaline phosphatase treated. The *H. holstii* mannan which had a mannose to phosphate ratio of 15 to 1 was a moderate inhibitor of the transfer reaction. The only acceptor was that associated with the catalytically active particulate fraction.

Substrate Specificity. Of the mannose-containing substrates tested, only GDP-mannose was active. [^{14}C]Mannose-1-P and [^{14}C]mannose-6-P are completely inactive (Table II, expt 1). Addition of a tenfold molar excess of unlabeled UDP-mannose reduced incorporation of [^{14}C]mannose from GDP-[^{14}C]mannose by only 6%; addition of IDP-mannose reduced incorporation by 46% and addition of GTP reduced incorporation by 10% (Table II, expt 2). Mannose itself has no effect on incorporation from GDP-[^{14}C]mannose.

Characterization of the ^{14}C Product. Essentially all of the [^{14}C]mannose incorporated into an ethanol-insoluble product remains associated with the particulate fraction. Total hy-



[a] Analysis by gas–liquid partition chromatography.

FIGURE 2: Fractionation scheme used for separation of the ^{14}C products. Several standard incubation mixtures were pooled and the particulate fraction, containing the bound ^{14}C products, was isolated and washed twice with H_2O by centrifugation. The suspended pellet was then extracted with butanol–pyridinium acetate and Tris buffer as described in Table I. The Tris buffer soluble and insoluble fractions were then fractionated as shown in this figure and as described more fully in the text. The numbers in parentheses are the per cent isotope recoveries from the starting ^{14}C-particulate fraction.

drolysis of this isotopic material yields mainly [^{14}C]mannose and smaller amounts of ^{14}C-labeled components which may be phosphate esters. In order to characterize this material more fully a solubilization and fractionation scheme was designed which would recover the maximum amount of high molecular weight radioisotope. The fractionation scheme involves extraction of the isolated ^{14}C-particulate fraction with butanol–pyridinium acetate to remove ^{14}C-lipid material and then partial solubilization of the remaining ^{14}C products with Tris buffer (see Table I). The Tris-insoluble products are solubilized by Pronase digestion. Small molecular weight ^{14}C material is then removed from the Tris-soluble and Tris-insoluble fractions by Sephadex G-50 chromatography. The Sephadex G-50 excluded, Tris-soluble ^{14}C products are further purified by precipitation with Fehling's solution and following Pronase digestion are fractionated on a DEAE-cellulose column. The Sephadex G-50 excluded, Tris-insoluble ^{14}C products are fractionated directly on a DEAE-cellulose column. A summary of this fractionation scheme including a percentage of the radioisotope recovered through each step is presented in Figure 2. The total amount of high molecular weight radioisotope isolated by this procedure is approximately 30% (the total recovery from the DEAE-cellulose columns), while 19% is solubilized by the lipid solvent and 50% is either of low molecular weight or not precipitated by Fehling's solution. The final products from DEAE-cellulose chromatography, when analyzed after acid hydrolysis by gas–liquid partition chromatography, were found to contain, as shown in Figure 2, mannose, glucose, and smaller amounts of unidentified components. Phosphate is also present in the material eluted at higher salt concentrations (Figure 3).

The Tris Buffer Solubilized Product. The ^{14}C products solubilized with Tris buffer (see Table I) were chromatographed on a Sephadex G-50 column. Approximately 70% of the applied ^{14}C material was excluded from the column. To an aliquot of the excluded material containing 9000 cpm in 0.1 ml of H_2O was added 3 mg of mannan followed by Fehling's solution. The precipitated material contained 6200 cpm. The product is also precipitated with Fehling's solution after autoclaving in 6 N NaOH for 2 hr.

Attempts to fractionate the Sephadex G-50 excluded material or the Fehling's-precipitable material on DEAE-cellulose (HCO_3^-) columns were unsuccessful as the radioisotope was not quantitatively eluted off at high (2 M) NH_4HCO_3 concentrations or with 1 M acetate buffer (pH 4.0). However, extensive Pronase digestion (40 hr) yielded a product which did fractionate on DEAE-cellulose (HCO_3^-) columns. As shown in Figure 3, the radioactive product fractionates into two major components which elute at approximately 0.06 M and 0.21 M NH_4HCO_3 and a minor component which elutes at 0.45 M NH_4HCO_3. Included in this figure are the positions at which Bakers yeast mannan, and *H. holstii* mannan, and phosphomannan elute. These mannans have mannose:phosphate molar ratios of >100:1, 15:1 and 5:1, respectively, and elute at approximately 0.09 M, 0.25 M, and 0.41 M NH_4HCO_3, respectively. Chemical analysis of the

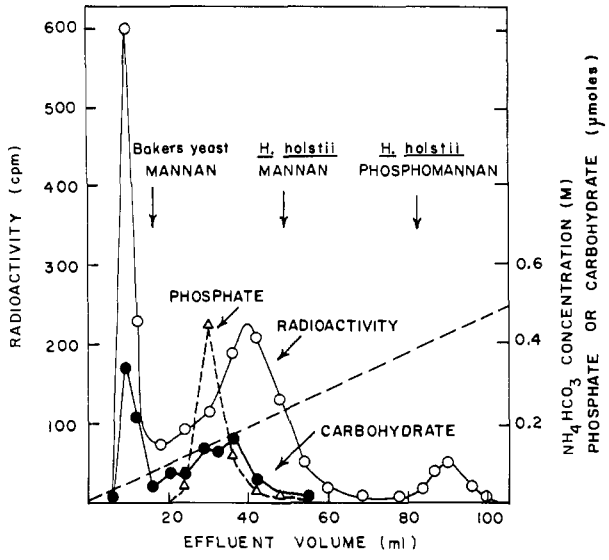


FIGURE 3: Elution pattern of the Tris buffer solubilized ^{14}C products from a DEAE-cellulose (HCO_3^-) column. The Sephadex G-50 excluded material was digested with Pronase and then applied to the DEAE-cellulose column. Fractions of 3 ml were collected. Radioactivity is expressed as cpm/0.3-ml aliquots counted in 10 ml of Bray's solution (Bray, 1960). Phosphate or carbohydrate is expressed as μmoles/3-ml fraction. The arrows mark the elution position of Bakers yeast mannan, *H. holstii* cell wall mannan, and *H. holstii* exocellular phosphomannan.

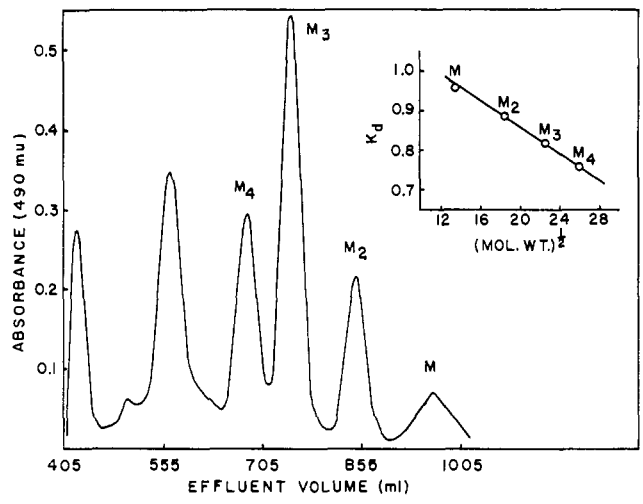


FIGURE 4: Elution pattern from a Sephadex G-15 column of oligosaccharides from acetolysis of *H. holstii* mannan. The mannan (0.1 g) was subjected to acetolysis (Lee and Ballou, 1965) and the deacetylated products were applied to a 3 × 185 cm column of Sephadex G-15 equilibrated with 0.1 M NH_4HCO_3. Fractions of 3 ml were collected after discarding the first 405 ml. Aliquots of 0.02 ml were analyzed for carbohydrate by the phenol–H_2SO_4 method.

DEAE-cellulose-fractionated ^{14}C material revealed varying amounts of carbohydrate throughout the first two peaks, whereas phosphate was absent in the first peak and present throughout the second peak. Analysis of the third minor component eluting at 0.45 M NH_4HCO_3 was not undertaken at this time.

The information obtained on the structures of yeast mannans using acetolysis under specified conditions which cleaves mainly 1→6 glycosidic linkages (Stewart and Ballou, 1968) indicated that this technique would be useful in characterization of the *in vitro* synthesized ^{14}C products. As shown in Figure 4, oligosaccharides are obtained from acetolysis of *H. holstii* mannan which are separated by Sephadex G-15 chromatography. The peaks labeled M,[2] M_2, M_3, and M_4 have the same paper chromatographic mobility in solvent C as do the corresponding compounds obtained from acetolysis of *S. cerevisiae* mannan (Lee and Ballou, 1965). A plot of K_d *vs.* $M_w^{1/2}$ (insert of Figure 4) gives a straight line as expected for a homologous series of oligosaccharides. The molar ratios, calculated from carbohydrate content as determined with phenol–H_2SO_4, are M, 0.51; M_2, 0.57; M_3, 1.00; M_4, 0.43. We therefore proceeded to use this technique even though the nature of the glycosidic linkages in these oligosaccharides had not been determined.

Acetolysis of ^{14}C product which had been solubilized with Tris buffer and excluded from Sephadex G-50 resulted in the production of [^{14}C]oligosaccharides through the tetramer which migrated with the *H. holstii* mannan oligosaccharide markers in solvent C (Figure 5). The per cent of the recovered radioisotope in each component was M, 22; M_2, 37; M_3, 20; M_4, 12; larger or not resolved, 9.

The tubes containing the DEAE-cellulose effluent volume from 6 to 18 ml (peak 1) and from 19 to 60 ml (peak 2) (Figure 3) were pooled separately and, after evaporation of the NH_4HCO_3, an aliquot of each containing 3000 cpm was submitted to acetolysis for 13 hr at 40°. The deacetylated products were separated by chromatography in solvent C and the distribution of radioactivity was determined (Table III). A large percentage of the recovered products is present as mannose and M_2, this being more pronounced in the acetolysis products of the material retarded longer on DEAE-cellulose (peak 2). Nevertheless, radioactive M_3 and M_4 were recovered as expected from degradation of *H. holstii* mannan.

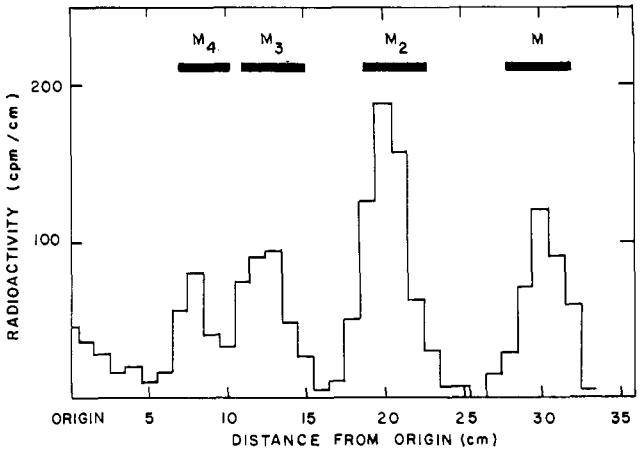


FIGURE 5: Paper chromatogram of ^{14}C-acetolysis products. The starting ^{14}C product was Tris solubilized and excluded from Sephadex G-50. Chromatography of the deacetylated oligosaccharides was carried out with solvent C for 12 hr, the paper cut into 1-cm strips, and counted in 10 ml of toluene cocktail.

[2] Abbreviations used are: M, mannose; M_2, M_3, M_4, di-, tri-, and tetrasaccharides of mannose.

TABLE III: Distribution of Acetolysis Products of Tris-Solubilized Material Fractionated on DEAE-Cellulose.[a]

	Distribution			
	Peak 1		Peak 2	
Product	cpm	%	cpm	%
M	664	29.7	1424	56.0
M_2	541	24.2	578	22.7
M_3	324	14.5	153	6.0
M_4	132	5.9	228	9.0
Larger[b]	576	25.7	163	6.3

[a] Peak 1 is 6–18 ml and peak 2 is 19–60 ml of the DEAE-cellulose fractionation shown in Figure 3. [b] This is ^{14}C material which migrates slower than M_4 and may also contain phosphate esters.

TABLE V: Distribution of Mild Acid Hydrolysis Products of Tris-Solubilized Material.

	Distribution (%)		
Fraction[a]	M	M_2	Origin
1			100
2			100
3	37	11	52
4	37	20	43

[a] The fractions were pooled from the effluent of a DEAE-cellulose column similar to that shown in Figure 3. Fraction 1 corresponds to 6–18 ml, fraction 2 to 19–30 ml, fraction 3 to 31–42 ml, and fraction 4 to 43–60 ml. Approximately 800 cpm of each fraction was hydrolyzed in 0.2 ml of 0.25 N HCl at 100° for 90 min. Solvent C was used for paper chromatography.

To determine if [^{14}C]mannose was present throughout these oligosaccharides or if addition from GDP-mannose was only to the nonreducing end of the acceptor molecule, representative areas of the chromatograms coresponding to M_2 and M_4 were eluted with H_2O and reduced with $NaBH_4$. Following acid hydrolysis, the samples were chromatographed in solvent F and the amount of radioactivity migrating with mannose and mannitol was determined by liquid scintillation counting (Table IV). The M_2 derived from peak 2 of the DEAE-cellulose chromatography contained equal amounts of radioactivity in mannose and mannitol. The M_2 derived from peak 1 contained almost four times as much radioactivity in mannose as in mannitol, indicating that [^{14}C]mannose was being transfered from GDP-[^{14}C]mannose to unlabeled acceptor molecules. Likewise, the products of the M_4 area of peak 1 exceeded the theoretical mannose:mannitol ratio of 3:1, although the observed 4.67:1.00 ratio indicates quite extensive incorporation of [^{14}C]mannose into all positions of the tetrasaccharide.

Other acid hydrolysis properties of the DEAE-cellulose-fractionated, Tris-solubilized product were examined. Total acid hydrolysis (1.3 N HCl, 100°, 3 hr), followed by evaporation of the HCl and chromatography in solvent C for 12 hr, revealed [^{14}C]mannose as the only hexose and a small amount (5%) of ^{14}C material which just moved off the origin. This slow-migrating material was not examined further. Mild acid hydrolysis (0.025 N HCl, 100°, 90 min) resulted in the release of mannose and M_2 from only the DEAE-cellulose-retarded fractions (Table V). The disaccharide was characterized as M_2 by chromatography in solvents C, D, and E, electrophoresis in 0.2 M borate buffer (pH 8.8), and degradation to [^{14}C]-mannose with α-mannosidase. The rates of release of mannose and M_2 with mild acid (Figure 6) indicate that the mannose is released at a much greater rate than is M_2 and is therefore probably not derived from M_2. The conditions of hydrolysis make it unlikely that the release of these components is due to the cleavage of glycosidic bonds. It is possible that phosphodiester bonds to the reducing end of the mannose and M_2 are being hydrolyzed, this being consistent with the retardations of the polymeric material on DEAE-cellulose. The presence of these acid-labile groups is also reflected in the acetolysis studies (Table III) where an abundance of mannose and M_2 was observed in the DEAE-cellulose-retarded fractions.

TABLE IV: Distribution of Radioactivity in Mannose Residues of Oligosaccharides from Acetolysis of Tris-Solubilized Product.

	Distribution		
Oligosaccharide[a]	Mannose, cpm	Mannitol, cpm	Ratio, Mannose:Mannitol
Peak 1, M_2	240	66	3.6:1.0
Peak 1, M_4	210	45	4.7:1.0
Peak 2, M_2	252	231	1.1:1.0

[a] The oligosaccharide obtained from acetolysis of the designated peaks from the DEAE-cellulose column (Figure 3) was eluted off the paper chromatogram and submitted to reduction and hydrolysis.

The Tris Buffer Insoluble Products. After solubilization of about 50% of the particle-bound radioactivity with mild alkali, the remainder of the radioactivity was solubilized by digestion with Pronase (80 hr). The solubilized material was passed through a Sephadex G-50 column as previously described. Only about 23% of the radioactivity was eluted at the void volume, the remainder eluting as a large broad peak near the void plus internal volume. The material emerging at the void volume was further fractionated on a DEAE-cellulose column into two components eluting at the same positions as shown for the Tris-solubilized material in Figure 3. There is a difference in the ratios of the two components, 80% of the radioactivity of the particle-bound material eluting in the second peak whereas about 50% of the Tris-solubilized material eluted in the second peak.

Mild acid hydrolysis (0.025 M HCl, 100°, 90 min) of these two components (800 cpm each) followed by chromatography in solvent C resulted in no breakdown of peak 1 material (all

TABLE VI: Distribution of Acetolysis Products of Pronase-Solubilized Product Fractionated on DEAE-Cellulose.

	Distribution			
	Peak 1		Peak 2	
Product	cpm	%	cpm	%
M	566	24.0	1279	57.8
M_2	924	39.1	441	19.9
M_3	485	20.6		
M_4	279	11.7		
Larger	105	4.6	495	22.3

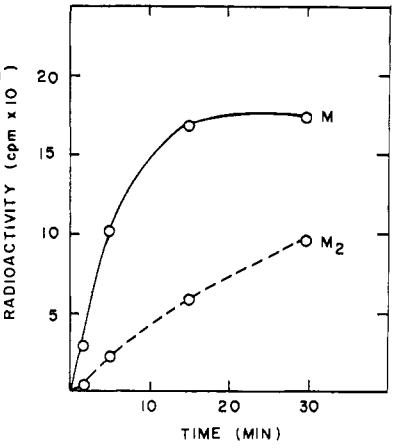


FIGURE 6: Rates of release of mannose and disaccharide from the product. Five standard incubation mixtures each contained 0.8 mg of particulate fraction protein and 1.9 mμmoles of GDP-[^{14}C]mannose. After a 30-min incubation, the mixtures were heat denatured and chromatographed in solvent A. The material at the origin was cut out and sealed in a test tube containing 4 ml of 0.01 N HCl. After hydrolyzing for the desired length of time in a boiling-water bath the reaction was stopped by adding sufficient NH_4HCO_3 to bring the pH to 7. The aqueous material was removed and the paper was washed three times with 0.5-ml portions of water. The washings and original material were pooled, reduced in volume, and chromatographed with solvent A. The radioactivity which migrated with oligosaccharides was eluted off the paper and chromatographed with solvent C. Radioactivity migrated with only mannose and mannobiose. This material was eluted off the paper and counted in Bray's scintillation solution: (O———O) mannose; (O---------O) disaccharide.

of the radioactivity remained at the origin), but did release 51 and 26% of the radioactivity as mannose and M_2, respectively, from peak 2 material.

The products of acetolysis of these two components resembles more closely the acetolysis products of peak 2 from DEAE-cellulose chromatography of the Tris-solubilized material in that a large percentage of the radioactivity is recovered as mannose and M_2 (Table VI). In fact, no distinct acetolysis pattern is observed for peak 2, as mild acid hydrolysis gave the same products in approximately the same ratio. The higher yield of mannose from acetolysis may reflect some breakdown of the disaccharide.

The ratios of radioactivity in mannose to mannitol derived from $NaBH_4$ reduction and acid hydrolysis of the disaccharides were 3.3:1.0 for peak 1 and 0.9:1.0 for peak 2. This is quite similar to the values obtained for the disaccharides from the Tris-solubilized product (Table IV).

Discussion

Evidence is presented that mannose is transferred from GDP-mannose to particle-bound acceptor molecules. Part of the [^{14}C]mannose-containing product resembles mannan in that it is of relatively high molecular weight, is precipitable with Fehling's reagent, and yields radioactive oligosaccharides of mannose after acetolysis. The isolation of radioactive oligosaccharides which contain [^{14}C]mannose throughout the molecule would imply that the synthesis we are observing is not just addition of a mannosyl residue to the nonreducing end of an acceptor molecule, but rather that several residues are being added. As detailed studies on the structure of *H. holstii* mannan have not yet been carried out, a comparison of the linkages in the ^{14}C-acetolysis products was not undertaken.

The fractionation scheme used in this work to isolate the ^{14}C products was designed to avoid chemical degradation. As shown in the fractionation scheme, some 37% of the particle-bound radioisotope is recovered as high molecular weight (excluded from Sephadex G-50). About 70% of this excluded material is precipitated with Fehling's reagent. Failure of the Tris-solubilized material to elute successfully from DEAE-cellulose unless digested with Pronase suggests that, up to this digestion step, it is a glycoprotein possibly rich in acidic amino acids. The extraction procedure used by Sentandreu and Northcote (1968) for the isolation of a yeast cell wall glycoprotein containing mannan is currently being considered in order to further clarify this point.

Fractionation of the Pronase-digested ^{14}C product into two components on DEAE-cellulose afforded further information as to the nature of the acceptor molecules. The Tris-solubilized product which is eluted from DEAE-cellulose at low salt concentrations (peak 1) appears to be mainly a neutral mannan, although traces of glucose are found. The particle-bound ^{14}C material which is not solubilized with Tris buffer but which is released with Pronase digestion also fractionates into two radioactive components, although in this case the material eluting at a higher salt concentration (peak 2) predominates. The material eluted at low salt concentration contains a great deal of glucose, even if previously precipitated with Fehling's reagent, and may represent a glucomannan or a mixture of glucan and mannan. The radioactive material which elutes from DEAE-cellulose at high salt concentration appears to be similar regardless of whether it was previously solubilized with Tris buffer or with Pronase. The elution position is indicative of an anionic molecule. Phosphate is present throughout these peaks and is probably the anionic group responsible for the elution position although the presence of acidic amino acids cannot be excluded at this time. The rapid release of [^{14}C]mannose and [^{14}C]mannobiose from this material after mild acid hydrolysis suggests the presence of a mannose 1-phosphoryl acceptor or a mannosyl-mannose 1-phosphoryl acceptor structure. The mannose units in this disaccharide appear to be preferentially incorporated from GDP-[^{14}C]mannose as the $NaBH_4$ reduction

and hydrolysis experiments indicated equal labeling of both residues.

The presence of phosphodiester linkages in yeast cell walls has been discussed by Lampen (1968) and preliminary evidence for a mannose 1-phosphoryl group in a yeast cell wall phosphoglycoprotein has been presented by Cawley and Letters (1968). What the linkage of the phosphoryl-acceptor moiety is remains uncertain as Stewart and Ballou (1968) have presented evidence for attachment of phosphate to the 3 or 4 position of mannose units in *Kloeckera brevis* mannan whereas Mill (1966) has reported the isolation of mannose 6-phosphate from *S. cerevisiae* mannan and Sentandreu and Northcote (1968) have isolated mannose 6-phosphate from Bakers yeast glycoprotein. Our experiments have been carried out with *H. holstii* which secretes a phosphomannan containing phosphodiester linkages between the carbon 1-hemiacetal position of one mannose unit and the carbon 6 position of another (Slodki, 1962). It is therefore possible that part of the ^{14}C product synthesized with the particulate fraction is exocellular phosphomannan, as earlier results were consistent with the synthesis of this polymer occuring in the cell membrane (Kozak and Bretthauer, 1968).

We have preliminary evidence that ^{32}P from [β-^{32}P]GDP-mannose is incorporated into this particulate enzyme fraction and after solubilization elutes from DEAE-cellulose with peak 2 ^{14}C product (Bretthauer *et al.*, 1969). Whether this represents the transfer of a mannose 1-phosphoryl unit from GDP-mannose is under investigation. In addition, the nature of the butanol pyridinium acetate soluble ^{14}C products is being studied as Tanner (1969) has presented evidence for a lipid intermediate in yeast mannan synthesis.

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