PHOSPHATE AND MANNOSE TRANSFER FROM GUANOSINE DIPHOSPHATE MANNOSE TO YEAST MANNAN ACCEPTORS[1]

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SUMMARY: A particulate enzyme isolated from Hansenula holstii catalyzes the transfer of ^{14}C-mannose from guanosine diphosphate ^{14}C-mannose and of ^{32}P-phosphate from β-^{32}P-guanosine diphosphate mannose to endogenous acceptor molecules. The ^{32}P-product is solubilized with Tris buffer, excluded from Sephadex G-50 and precipitated with Fehling's reagent. ^{32}P-Mannose 6-phosphate is recovered from acid hydrolyzates. Mild acid hydrolysis liberates newly incorporated mannose residues and allows the ^{32}P-phosphate to be released as inorganic phosphate by phoshomonoesterase. The synthesis of a 1,6'-phosphodiester linkage between 2 mannose residues is proposed.

We have initiated studies on the biosynthesis of mannans with the yeast Hansenula holstii which, in addition to having a relatively highly phosphorylated wall mannan, produces an exocellular phosphorylated mannan (mannose: phosphate ratio of 5:1) containing mannose 1→phosphate→6 mannose linkages (1). A particulate enzyme fraction has been isolated which actively catalyzes the transfer of ^{14}C-mannose from GDP - ^{14}C-mannose to endogenous mannan acceptors (2). The same particulate enzyme fraction catalyzes the transfer of ^{32}P-phosphate from β-^{32}P-GDP-mannose to particle-bound acceptors. This report summarizes our results concerning

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the properties of this ^{32}P-phosphate transfer reaction and the nature of the product formed.

Materials and Methods - GDP-^{14}C-mannose and ^{32}P-inorganic phosphate were obtained from New England Nuclear. The β-^{32}P-GDP-mannose was synthesized enzymatically (3) with the use of γ -^{32}P-ATP (4). ^{14}C-Mannose-6-P and ^{32}P-mannose-6-P were prepared with hexokinase. ^{32}P-Mannose-1-P and ^{14}C-mannose-1-P were prepared from the respective labeled GDP-mannose by cleavage with a yeast nucleotide pyrophosphatase (5). β-^{32}P-GDP was obtained from the sugar nucleotide by mild acid hydrolysis.

H. holstii NRRL-Y 2448 was grown as described by Anderson et al. (6) with 2% glucose on a gyrotory shaker for 24 hr to late log phase. The harvested cells were washed twice with 1% KCl and then with 0.025 M imidazole acetate buffer pH 6.5 containing 0.001 M mercaptoethanol. The cells were suspended in the same buffer and ruptured by passing through a French Pressure Cell at 7000 psi. The broken cell suspension was centrifuged for 5 min at 1000g and the supernatant fluid obtained recentrifuged. The cell-free supernatant fluid was then centrifuged at 75000g for 45 min and the pellet obtained was washed three times by suspension and centrifugation in the same buffer. The washed, resuspended particulate fraction was stored in liquid nitrogen.

Incubation mixtures contained in 0.1 ml: 25 mM imidazole acetate pH 6.5; 10 mM $MnCl_2$; 0.2-0.5 mg particulate fraction protein; 10 mM GDP-mannose (1-10 μCi/μmole). The reaction was terminated after 30 min at 30° by placing the tube in boiling

water for 2 min. Routine assays involved chromatography of the entire heat denatured mixture in solvents A or C and determining the amount of radioisotope remaining at the origin of the chromatogram.

All paper chromatography was carried out on Schleicher and Schuell No. 589 Green Ribbon C paper in the descending direction with the following solvents: A, 95% ethanol: 1 M ammonium acetate pH 7.5 (7.3v/v); B, ethanol: methyl ethyl ketone: 0.5 M morpholinium tetraborate, pH 8.6, in 0.01 M EDTA (7:2:3 v/v) (with papers previously dipped in 0.01 M EDTA, pH 7.0) (7); C, isobutyric acid: 1 M NH_4OH (5:3v/v). Paper electrophoresis was conducted with acetic acid washed Whatman No. 3MM paper and 0.2 M borate buffer pH 8.8 at 30 volts/cm for 1 hr.

Results and Discussion - The incorporation of radioactivity from β -^{32}P-GDP-mannose and GDP-^{14}C-mannose into material which remains at the origin after chromatography in solvent A is time dependent. The molar ratio of the two radioactive groups incorporated is approximately 7 and invarient with time. The rate and extent of incorporation of either ^{32}P-phosphate or ^{14}C-mannose is dependent on both substrate and particulate fraction protein concentration. Divalent metal ions are required, $Mn^{\#}$ being the most effective at 25 mM for ^{32}P-phosphate transfer and 10 mM for ^{14}C-mannose transfer. The incorporation of both ^{32}P-phosphate and ^{14}C-mannose is inhibited 70% by either 0.5 mM GMP or GDP. No incorporation of radioisotope occurs with ^{32}P-mannose-6-P, β-^{32}P-GDP, ^{32}P-mannose-1-P, ^{14}C-mannose-6-P or ^{14}C-mannose-1-P.

For characterization of the ^{32}P-product, the standard incubation mixture was scaled up 20 times (1.1×10^7 cpm of ^{32}P). After

incubation for 40 min at 30°, the mixture was extracted with butanol-6 M pyridinium acetate pH 4.2 (4:1v/v) and the residue in the aqueous phase collected by centrifugation. The washed residue was suspended in 0.1 M Tris buffer pH 8.0 and heated at 100° for 30 min. The soluble material, collected by centrifugation, contained 1.3×10^6 cpm of ^{32}P-material. After passing through a Sephadex G-50 column(1 x 100 cm in 0.1 M NH_4HCO_3), 5.3×10^5 cpm of ^{32}P-material emerged at the void volume. Fehling's reagent (8) precipitated 49% of the ^{32}P-material and upon reprecipitation, 96% of the radioisotope was recovered. After digestion with Pronase (Calbiochem) the product was retarded on a DEAE-cellulose [HCO_3^-] column and, after applying a gradient of NH_4HCO_3, eluted at a position

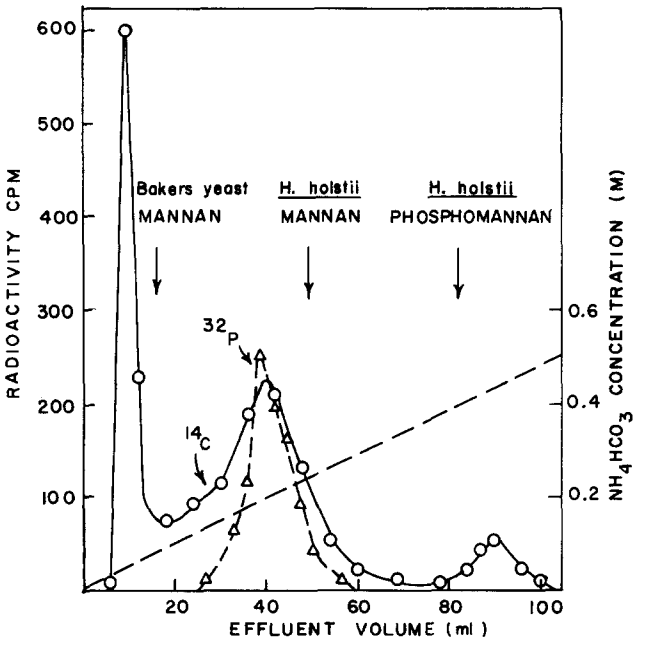

Fig. 1 Elution pattern of ^{14}C-and ^{32}P-products from a DEAE-cellulose [HCO_3^-] column. Both products were Tris buffer solubilized and excluded from Sephadex G-50 prior to chromatography. The column was 1 x 10 cm. The arrows indicate the peaks of the elution curves for bakers yeast mannan, H. holstii cell wall mannan, and H. holstii exocellular phosphomannan (mannose: phosphate ratios of 100:1, 18:1 and 5.1 respectively).

where part of the ^{14}C-product (Tris buffer solubilized, Sephadex G-50 excluded and precipitable with Fehling's reagent) also elutes and where H. holstii wall mannan elutes (Fig. 1). Hydrolysis of the Sephadex G-50 excluded ^{32}P-product in 1 N HCl for 2.5 hr at 100° followed by addition of carrier mannose-6-P and isolation of the water soluble, ethanol insoluble barium salts resulted in recovery of 80% of the ^{32}P. All

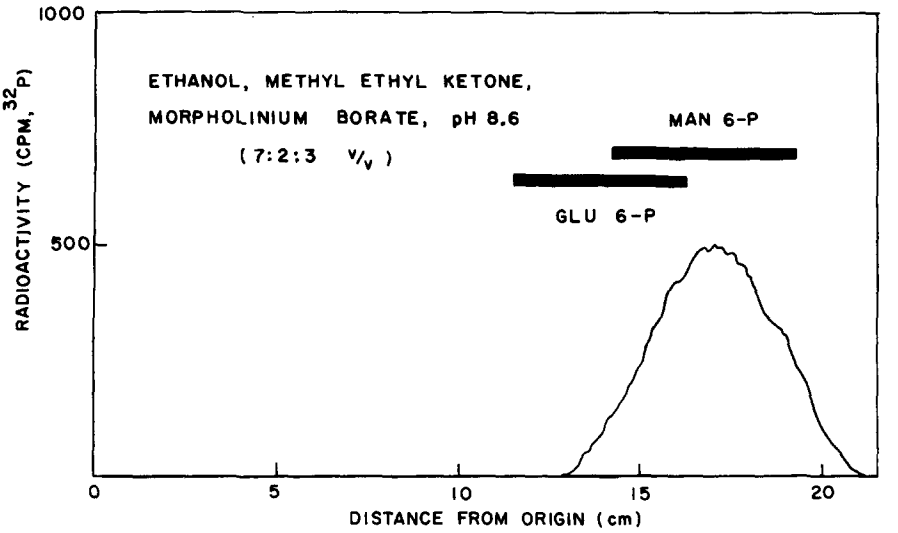

Fig. 2 Paper chromatogram of acid hydrolysis products from ^{32}P-product. The chromatogram was irrigated with solvent B for 9 hr, dried and scanned with a Packard radiochromatogram scanner.

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of this radioactivity migrated with mannose-6-P in solvent B (Fig. 2) and after reduction with sodium borotritiide, the ^{32}P and ^{3}H co-migrated with mannitol-6-P (Fig. 3). Periodate oxidation of the ^{32}P-hexose phosphate and of mannose-6-P, followed by chromatography in solvent B or electrophoresis resulted in co-migration of the ^{32}P with the phosphate positive component (7) from mannose-6-P which is presumable glycoaldehyde phosphate

Other studies have shown that a large percentage of the ^{14}C-product which elutes with the ^{32}P-product from DEAE-cellulose is hydrolyzed

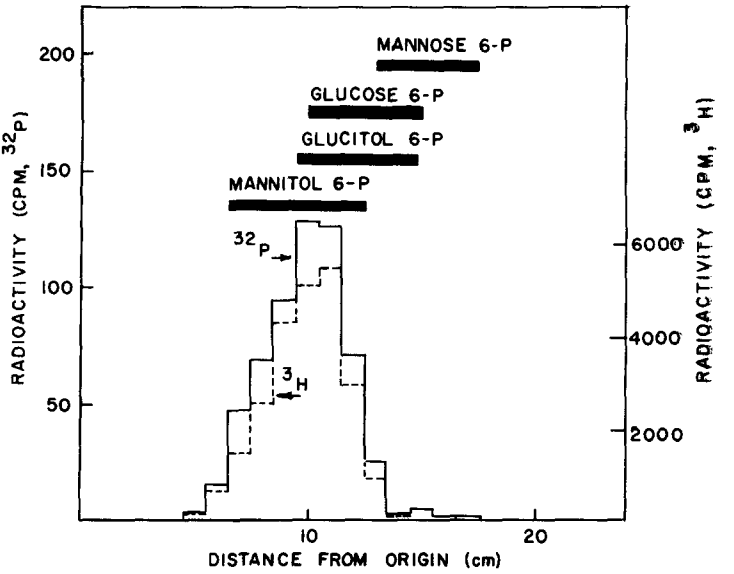

Fig. 3 Paper chromatogram of sodium borotritiide reduced, acid hydrolysis products from ^{32}P-product. The chromatogram was irrigated with solvent B for 8 hr, dried, and cut into 1 cm strips for counting in the Packard Tri-Carb spectrometer.

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with mild acid (0.01 N NCl, 100°, 30 min) to yield ^{14}C-mannose and ^{14}C-mannosyl-mannose (2). As this is indicative of an acid labile phosphate ester linkage, the ^{32}P-product was subjected to phosphomonoesterase treatment before and after mild acid hydrolysis. As shown in Table I, acid hydrolysis or phosphatase treatment alone resulted in the release of only small amounts of inorganic phosphate, whereas prior mild acid hydrolysis allowed about 30% of the ^{32}P to be released as inorganic phosphate by phosphatase.

We interpret these results to mean that mannose-1-P is being trasferred from GDP-mannose to endogeneous acceptor molecules (mannan or a glycoprotein). Heating in Tris buffer solubilizes a large percentage of the newly synthesized material which is of large molecular weight and resembles mannan in that it is precipitated with Fehling's reagent.

Table I

Release of Inorganic Phosphate by Phosphatase after Mild Acid Hydrolysis

Additions[1]	Time of Acid Hydrolysis (min) 0	20	40
		cpm	
Acid Alone[2]	0	16	80
Phosphatase Alone[3]	44	-	-
Acid, then Phosphatase	-	1012	1140

[1] Each experiment contained 4000 cpm of ^{32}P-product.
[2] Hydrolysis was carried out in 0.05 NHCl at 100°.
[3] Incubations were carried out at pH 8.0 at 30° for 21 hr with 100 µg/ml alkaline phosphatase.

The acceptor molecules have mannose residues at the nonreducing end which may originate from the added GDP-mannose or may be contained within the particulate fraction as isolated. Strong acid hydrolysis liberates this acceptor mannose residue, with the attached, newly incorporated phosphate residue, as mannose-6-P. That this phosphate is present in the polymer in phosphodiester linkage is evidenced by its susceptibility to phosphomonoesterase.

Further studies are underway to further clarify these results and to determine if the newly synthesized material is cell wall mannan (or glycoprotein (9)), or exocellular phosphomannan.

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