Characterization of Phosphorylated Oligomannosides from *Hansenula wingei* Mannoprotein†

Carl Hashimoto, Robert E. Cohen,‡ and Clinton E. Ballou*

ABSTRACT: Yeast cell wall mannoproteins often contain phosphate esterified to the oligosaccharide side chains, and partial acetolysis yields both neutral and phosphorylated fragments [Thieme, T. R., & Ballou, C. E. (1971) *Biochemistry 10*, 4121–4129]. We have isolated the acidic acetolysis fragments from *Hansenula wingei* mannoprotein [Yen, P. H., & Ballou, C. E. (1974) *Biochemistry 13*, 2420–2427] and have separated them into a mannopentaose monophosphate (Man_5P) and a mannotriose monophosphate (Man_3P). On the basis of selective enzymatic and partial acid hydrolysis and ^1H and ^{31}P NMR studies, Man_5P was shown to have the structure P→6αMan1→3αMan1→2αMan1→2αMan1→2Man (where Man = D-mannopyranose). The ^{31}P NMR spectrum of the Man_3P showed it to be a mixture of a phosphate monoester and a phosphate diester, which was separated by ion-exchange chromatography. The monoester had the structure P→6αMan1→3αMan1→3Man whereas the diester had the properties of a cyclic phosphate. Although native *H. wingei* mannoprotein contains phosphodiester linkages, the starting mannoprotein preparation was isolated under alkaline conditions that hydrolyze such bonds, and it did not show a phosphodiester signal in the ^{31}P NMR spectrum. We conclude that the cyclic phosphate was an artifact formed during the acetolysis reaction. Because acetolysis of *H. wingei* mannoprotein yields only phosphorylated mannotriose and mannopentaose whereas the mannoprotein contains mannotetraose side chains as well, the phosphorylation process must be a very specific event in the biosynthesis of the glycoprotein.

Most yeast cell walls are composed of a fibrous glucan network interspersed with a matrix of mannoproteins of various types (Ballou, 1976). Among these extracellular glycoproteins are the enzymes invertase and acid phosphatase and macromolecules involved in sexual recognition and agglutination (Crandall & Brock, 1968). The latter are formed by haploid cells of perfect yeasts, and their synthesis is normally repressed in heterozygous diploids (Manney & Meade, 1977). In previous studies on the sexual agglutinin of the yeast *Hansenula wingei* (Yen & Ballou, 1974a,b), we have shown that this glycoprotein contains about 5% phosphate in a diesterified form. Mild acid hydrolysis released glucose and mannose, and the conditions used suggest that these hexoses were attached as glycosyl phosphates units. During this treatment, the phosphate remained linked to the mannoprotein, and complete acid hydrolysis then yielded mannose 6-phosphate. These results indicate that glycosyl phosphate units were attached to position 6 of mannose residues in the mannoprotein. Similar structures have been found in other yeasts (Thieme & Ballou, 1971; Rosenfeld & Ballou, 1974) whose mannoproteins differ in structure from that of *H. wingei*.

In this study, we set out to determine the precise location of the phosphate in *H. wingei* mannoprotein by characterizing the phosphorylated oligosaccharides that are released by partial acetolysis (Lee & Ballou, 1965). The major products were a phosphorylated pentamannoside and a phosphorylated trimannoside. In both compounds, the phosphate was located on position 6 of the nonreducing terminal mannose unit, but the trimannoside phosphate contained some isomeric material in which the phosphate group was cyclized. It is probable that this material was formed during the acetolysis reaction because a phosphodiester signal was not apparent in the ^{31}P NMR spectrum of the mannoprotein.

An interesting sidelight to the characterization of these compounds is the recent evidence that phosphorylated mannose oligosaccharides may be sites of recognition for the uptake of lysosomal enzymes by mammalian cells (Distler et al., 1979). Whether they have a related function in yeast is unknown.

Experimental Procedures

Materials. Dowex AG 1-X2, Dowex AG 50-W-X8, Bio-Gel P-2 (−400 mesh), and Bio-Gel P-4 (−400 mesh) were from Bio-Rad; Sephadex G-10 and DEAE-Sephadex A-25 were from Pharmacia. Sigma was the source for barium mannose 6-phosphate, disodium fructose 6-phosphate, sodium glucose 6-phosphate, yeast mannosephosphate isomerase (EC 5.3.1.8), yeast glucosephosphate isomerase (EC 5.3.1.9), *Torula* yeast glucose-6-phosphate dehydrogenase (EC 1.1.1.49), and jack bean α-mannosidase (EC 3.2.1.24). Disodium $NADP^+$ came from Boehringer Mannheim, sodium [^3H]borohydride (200 Ci/mol) from New England Nuclear, and bacterial alkaline phosphatase (EC 3.1.3.1, BAPC grade) from Worthington. Mannan from *H. wingei* NRRL Y-2340 type 5 yeast was isolated by precipitation with Fehling's solution (Peat et al., 1961) or as a borate complex with Cetavlon (Lloyd, 1970). D-Mannose 4,6-phosphate was prepared from D-mannose 6-phosphate with dicyclohexylcarbodiimide (Khorana et al., 1957). The cyclic phosphate of *H. holstii* Man_5P was prepared similarly.

General Methods. Carbohydrate was assayed by the phenol–sulfuric acid method (Dubois et al., 1956) and phosphate by the method of Ames & Dubin (1960). Descending chromatography on Whatman No. 1 filter paper was done in solvent systems (A) ethyl acetate/pyridine/water (10:4:3) and (B) ethyl acetate/pyridine/water (5:3:2). Sugars and sugar alcohols were detected with the silver nitrate/sodium hydroxide dip reagent (Trevelyan et al., 1950) and radioactivity was detected with a Packard Model 7201 radiochromatogram scanner. α-Mannosidase digestions were done in 50mM sodium acetate, pH 4.5, at 37 °C, and alkaline phosphatase digestions in 0.1 M ammonium bicarbonate, pH 8.5, at 37 °C.

Acetylation and acetolysis of mannan were done according to Kocourek & Ballou (1969) with a modification to isolate

† From the Department of Biochemistry, University of California, Berkeley, California 94720. *Received May 23, 1980.* This work was supported by National Science Foundation Grant PCM 77-27334 and U.S. Public Health Service Grant AI-12522.

‡ Present address: Department of Molecular Biology, University of California, Berkeley, CA 94720.

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phosphorylated oligosaccharides (Karson & Ballou, 1978).

Sodium [^{3}H]borohydride reduced phosphorylated oligosaccharides were hydrolyzed in 0.10 N HCl for 60 min or 0.33 N HCl for 40 min, both at 100 °C. Under these conditions, 30% of the initial radioactive material was converted from an acidic to neutral form as determined by the procedure described below. After the HCl was removed under vacuum, the hydrolyzed sample was dissolved in water and adsorbed to a 1-mL column of DEAE-Sephadex A-25 (HCO_3^-). The column was washed with water until all neutral radioactive material was eluted, and then it was eluted with 0.5 M ammonium bicarbonate to release all bound radioactive material. The neutral effluent was concentrated and spotted on paper for chromatography. The acidic effluent was concentrated to dryness, dissolved in 0.2 mL of 0.1 M ammonium bicarbonate, pH 8.5, and incubated with 0.04 unit of alkaline phosphatase at 37 °C for 12 h. The reaction was terminated by boiling it, and the product was passed through a 1-mL column of Dowex AG 1 ($CH_3CO_2^-$) and Dowex AG 50 (H^+) by elution with water until all of the radioactive dephosphorylated material was collected. The effluent was concentrated and analyzed by paper chromatography.

Mannose 6-Phosphate Assay. This coupled enzyme assay is a modification of one described by Gawehn (1974). The sample, containing 4–10 nmol of organic phosphate, was hydrolyzed in 1 N trifluoroacetic acid at 120 °C for 2 h in a sealed glass tube. After evaporation of the acid under a N_2 stream, 1 mL of reagent containing $MgCl_2$ (10 mM), $NADP^+$ (200 μM), glucose-6-phosphate dehydrogenase (1.8 units), glucosephosphate isomerase (1.9 units), mannosephosphate isomerase (0.96 unit), and 80 mM Tris-HCl, pH 8.0, was added to the hydrolyzed sample. The conversion of mannose 6-phosphate to 6-phosphogluconate, coupled to the production of NADPH, was allowed to go to completion (60 min at 25 °C). NADPH production was measured with a Spex fluorometer (λ_{ex} = 340 nm, λ_{fl} = 458 nm).[1] A mannose 6-phosphate standard was treated in the same manner. The hydrolyzed sample was also incubated with the reagent lacking the mannosephosphate isomerase or both isomerases in order to detect fructose 6-phosphate and glucose 6-phosphate, respectively.

NMR Spectroscopy. ^{1}H spectra were obtained at 180 MHz and ^{31}P spectra at 72.9 MHz on an instrument equipped with a Bruker superconducting magnet and Nicolet 1180 computer operated in the Fourier-transform mode with quadrature detection. Data were accumulated into 8192 addresses, and a spectral width of 1500 Hz was used routinely.

Proton spectra were acquired at 40.0 ± 0.2 °C, using an 80° pulse width and 3.1-s cycle time. A digital broadening from 0.05 to 0.20 Hz was applied to enhance the sensitivity. Each sample was prepared by passing the aqueous solution through a 1-mL column of Chelex 100 (Na^+ form) to remove divalent metal ions, exchanging it 3 times with D_2O by lyophilization, and dissolving the product in 100.0% D_2O (Aldrich, low in paramagnetic ions grade). Proton chemical shifts are referenced indirectly to sodium 3-(trimethylsilyl) propanesulfonate with an internal acetone standard (δ 2.217 at 40.0 °C). Concentrations ranged from 0.1 to 2.0 mM, and 5-mm sample tubes were used.

^{31}P NMR spectra were obtained at a probe temperature of 25.0 or 30.0 ± 0.2 °C, with a 70° pulse width and 3.2-s cycle time. A digital broadening of 0.20 or 0.50 Hz was used. For broad-band ^{1}H decoupling, a bilevel decoupling power scheme of 2.5 W during the free-induction decay acquisition and 0.5 W during delay times was employed to minimize sample heating. Mannan samples were 10% in 0.1 M sodium (ethylenedinitrilo)tetraacetate, pH 7.0, buffer containing 10% D_2O to serve as the field-frequency lock signal, and 10-mm sample tubes were used. Oligosaccharides, after metal ion removal with Chelex as described above, were dissolved in the same buffer and transferred to 450-μL microcells (Wilmad Glass Co.) for spectroscopy. ^{31}P chemical shifts are referenced to external 85% orthophosphoric acid.

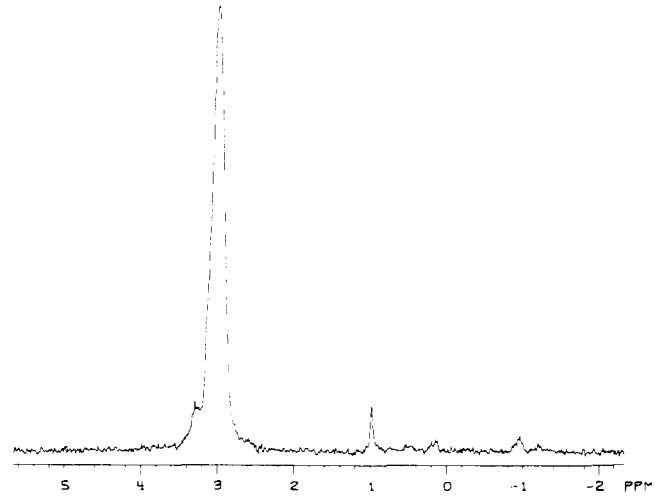

FIGURE 1: ^{31}P NMR spectrum of *H. wingei* mannoprotein isolated by Fehling's precipitation. The spectrum was taken at pH 6.3, and the signal at 3 ppm is characteristic of a phosphate monoester. No phosphate diester signal is apparent, and this is attributed to degradation by the alkaline reagent.

Results

H. wingei Y-5 Mannan Contains Mannose 6-Phosphate. H. wingei cells bind Alcian Blue dye strongly, which is evidence for the presence of phosphate in the cell wall (Friis & Ottolenghi, 1970). Mannan isolated from *H. wingei* 5 cells by Fehling's precipitation had a mannose to phosphate molar ratio of 20; the inorganic phosphate content of this preparation was 3% of the total phosphate. The ^{31}P NMR spectrum of the Fehling's-precipitated mannan displayed a major signal in the region characteristic of monoesterified phosphate and a faint signal in the region of inorganic phosphate, but no signal corresponding to diesterified phosphate (Figure 1). In contrast, the spectrum of mannan isolated by the Cetavlon method, which unlike the former procedure does not involve alkali, showed two signals of roughly equal intensity appearing in the phosphomonoester region (about 3 ppm at pH 6.3) and phosphodiester region (about −1 ppm at pH 6.3). The Fehling's-precipitated mannan was used in all subsequent experiments.

For unambiguous identification of the phosphorylated sugar as mannose 6-phosphate, an aliquot of the total acid hydrolysate of Y-5 mannan was assayed by the coupled enzyme system in which the sugar phosphate is isomerized to glucose 6-phosphate and oxidized by $NADP^+$ to gluconate 6-phosphate and NADPH. NADPH production, equivalent to the amount of organic phosphate initially added, was observed when all three enzymes were present in the incubation mixture; no reaction occurred when mannosephosphate isomerase was absent (Table I). Thus, phosphate is attached to position 6 of a mannose residue in Y-5 mannan.

Acetolysis of Y-5 Mannan Produces Two Major Phosphorylated Oligosaccharides. Y-5 mannan was acetolyzed

[1] Abbreviations used: Y-5, *H. wingei* type 5 cell; r, reduced; λ_{ex} and λ_{fl}, excitation and fluorescence emission wavelengths.

Table I: Assays for Mannose 6-Phosphate

sample	organic P added (nmol)	NADPH produced (nmol)	NADPH/P
Y-5 mannan	6.0	5.8, 6.0	0.98
Man_5P	6.0	5.6, 5.7	0.94
Man_3P-A	6.0	1.1, 1.0	0.18
Man_3P-B	6.0	6.8, 6.5	1.1

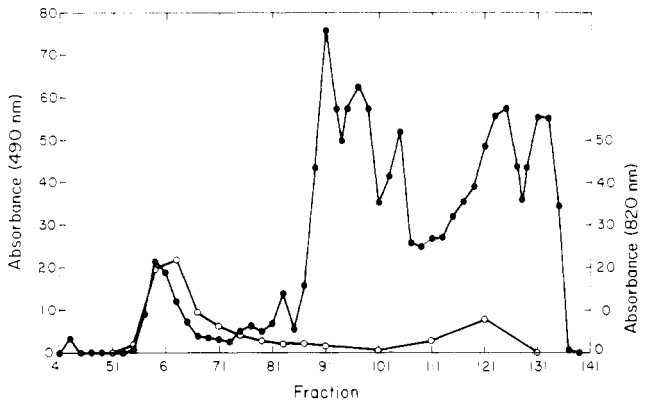

FIGURE 2: Fractionation of the acetolysis products of *H. wingei* Y-5 mannoprotein. The mixture of oligosaccharides was separated on a Bio-Gel P-2 column (2 × 190 cm) by elution with water. The phosphorylated fragments were eluted first (fractions 53–73), followed by the neutral oligosaccharides. A_{490} is carbohydrate (closed circles) and A_{820} is phosphate (open circles). Fractions of 4 mL were collected.

under conditions wherein the 1→6 glycosidic bonds are cleaved preferentially (Lee & Ballou, 1965) to obtain phosphorylated oligosaccharides. The acetolysis products were deacetylated and then chromatographed on either a Bio-Gel P2 column (2 × 190 cm) (Figure 2) or a Bio-Gel P4 column (4 × 190 cm) with water as the eluant. This resulted in the separation of phosphorylated fragments from the five neutral oligosaccharide products previously reported (Yen & Ballou, 1974a). Acid hydrolysis of the phosphorylated material gave products corresponding to mannose and mannose 6-phosphate, as revealed by paper chromatography in solvent system A (not shown).

The phosphorylated fragments were fractionated on a Bio-Gel P2 column (2 × 190 cm) equilibrated with 0.2 M ammonium acetate. Two major phosphorylated oligosaccharides with elution properties of a mannopentaose phosphate (Man_5P) and a mannotriose phosphate (Man_3P) that represented 15% of the phosphate of the mannoprotein sample were found (Figure 3). The Man_5P and Man_3P samples were rechromatographed and then freed from neutral contaminants by adsorption to 1-mL columns of DEAE-Sephadex A-25, which were washed with water and eluted with 0.5 M ammonium bicarbonate. Samples were desalted by gel filtration on a Sephadex G-10 column (1 × 25 cm) in water. We obtained 12 mg of Man_5P and 10 mg of Man_3P from the acetolysis of 3.4 g of Y-5 mannan. The Man_5P had a mannose to phosphate molar ratio of 5.6 and Man_3P had a ratio of 4. Subsequent analyses of Man_3P gave ratios closer to 3.

The ^{31}P NMR spectra of Man_5P and Man_3P both had a signal in the phosphomonoester region (Costello et al., 1975) which coincided with the signal produced by standard mannose 6-phosphate (not shown). This signal was split into a triplet in the proton-coupled spectra, which indicated that the phosphate in Man_5P and Man_3P was esterified to a primary hydroxyl (Thieme & Ballou, 1971; Costello et al., 1975). In addition to the phosphomonoester signal in the proton-decoupled ^{31}P NMR spectrum of Man_3P, there were two signals

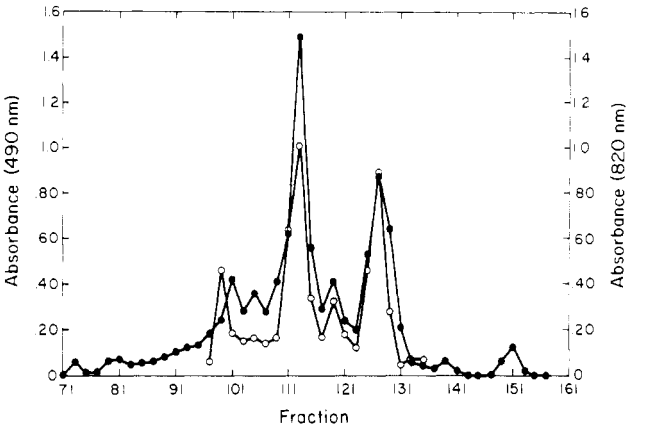

FIGURE 3: Isolation of phosphorylated mannotriose and mannopentaose. The oligosaccharide phosphate fraction from Figure 2 was fractionated on a Bio-Gel P-2 column (2 × 190 cm) by elution with 0.2 M ammonium acetate. Fractions of 3 mL were collected. The Man_5P was eluted first (fractions 110–118) and the Man_3P second (fractions 122–131). A standard phosphorylated mannotriose (Thieme & Ballou, 1971) was eluted in fractions 121–127. A_{490} is carbohydrate (closed circles), and A_{820} is phosphate (open circles).

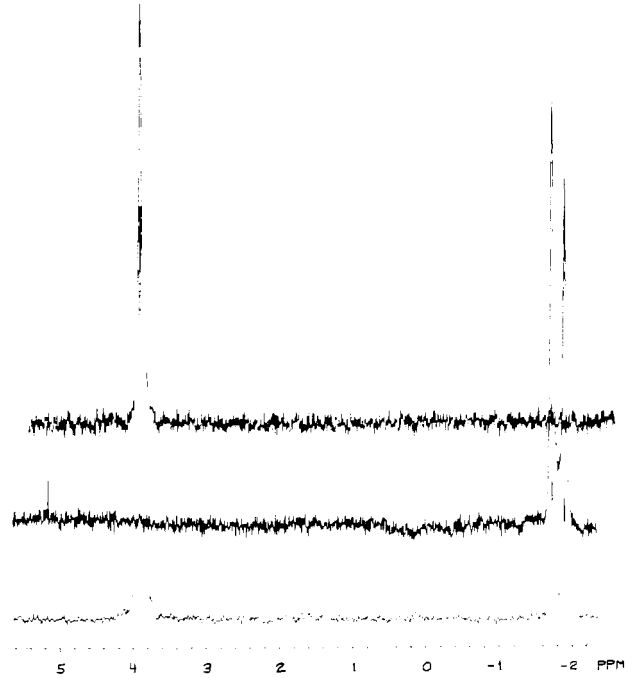

FIGURE 4: ^{31}P NMR spectra of mannotriose phosphate preparations. The bottom tracing is for the Man_3P preparation obtained by gel filtration in the presence of salt, corresponding to fractions 122–131 in Figure 3. Ion-exchange chromatography of this material yielded two peaks (Figure 5), the middle spectrum corresponding to peak A and the top spectrum to peak B. The spectra were measured at pH 7.0, and the signal at 4 ppm is characteristic of a phosphate monoester, whereas those at −2 ppm are characteristic of cyclic phosphate diesters such as mannose 4,6-phosphate. The two signals at −2 ppm suggest a mixture of two isomers. The different chemical shifts for monoester phosphate in this figure and Figure 1 result from the differences in pH.

further upfield in the region characteristic of diesterified phosphates (Figure 4). Similar upfield signals were not observed in the proton-decoupled spectrum of Man_5P. This suggested that Man_3P, but not Man_5P, was a mixture of phosphorylated oligosaccharides. Ion-exchange chromatography on DEAE-Sephadex A-25 resolved Man_3P into two components, labeled Man_3P-A and Man_3P-B (Figure 5). When observed with ^{31}P NMR spectroscopy, we found that Man_3P-B gave only the one signal in the phosphomonoester

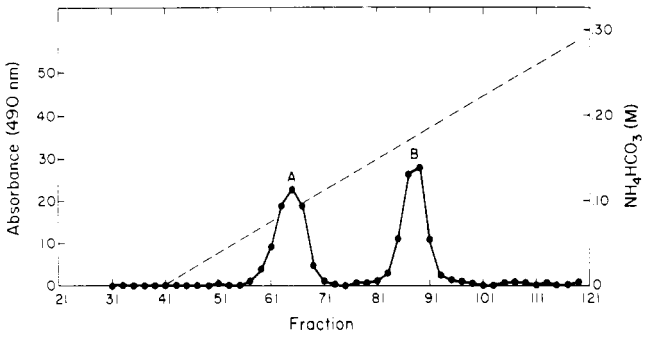

FIGURE 5: Separation of monoester and diester forms of Man_3P. Ion-exchange chromatography was done on a DEAE-Sephadex A-25 column by elution with a linear gradient of 0–0.5 M ammonium bicarbonate. A_{490} is carbohydrate. Fractions of 0.25 mL were collected.

region, whereas Man_3P-A produced the two signals in the phosphodiester region (Figure 4). Man_3P-A had a mannose to phosphate molar ratio of 3.4 and Man_3P-B had a ratio of 2.7.

Because a phosphodiester signal was not evident in the ^{31}P NMR spectrum of the mannan used in our study (Figure 1), this suggested that the phosphate in Man_3P-A would have been cyclized to a diester during the isolation procedure. Exposure of mannose 6-phosphate to similar acetolysis conditions did not, however, generate a cyclic phosphodiester. The ^{31}P NMR spectrum of synthetic mannose 4,6-phosphate (Khorana et al., 1957) and of *H. holstii* Man_5P cyclic phosphate similarly prepared showed only one signal, and it coincided with the high-frequency signal from Man_3P-A (not shown). Further characterization was unsuccessful owing to the limited amount of material available.

The phosphorylated sugar in Man_5P and Man_3P-B, as in Y-5 mannan, is mannose 6-phosphate. After acid hydrolysis, Man_5P and Man_3P-B gave products that yielded NADPH when incubated in the coupled enzyme assay with NADP. Essentially all of the organic phosphate added to the incubation mixture was converted to gluconate 6-phosphate (Table I). No fructose 6-phosphate or glucose 6-phosphate was detected when mannosephosphate isomerase was left out of the assay. Acid-hydrolyzed Man_3P-A, however, produced NADPH equivalent to only 18% of the added organic phosphate. If the phosphate in Man_3P-A is cyclized, it seems that only a small fraction can be converted to mannose 6-phosphate on acid hydrolysis. Stability in acid and alkali is a characteristic property of six-membered cyclic phosphates such as mannose 4,6-phosphate (Khorana, 1961).

Sodium borotritide reduced Man_5P (^3H-rMan_5P) and Man_3P-B (^3H-rMan_3P-B) were adsorbed to 1-mL columns of DEAE-Sephadex A-25, and elution was performed first with water and then with 0.5 M ammonium bicarbonate. Paper chromatography in solvent system B was used to analyze the neutral and acidic effluents, the latter after alkaline phosphatase treatment. The neutral effluent of ^3H-rMan_5P did not contain any radioactive material, but the acidic effluent contained radioactive reduced mannopentaose (Figure 6). Similar treatment of ^3H-rMan_3P-B yielded no neutral radioactive material, but a radioactive reduced mannotriose was produced by phosphatase treatment of the acidic fraction (Figure 7). Thus, in agreement with the estimations based upon gel filtration, Man_5P was a mannopentaose phosphate and Man_3P-B was a mannotriose phosphate. The radioactive material in the acidic effluent of ^3H-rMan_3P-A was resistant to alkaline phosphatase action. No radioactivity was detected in the neutral effluent.

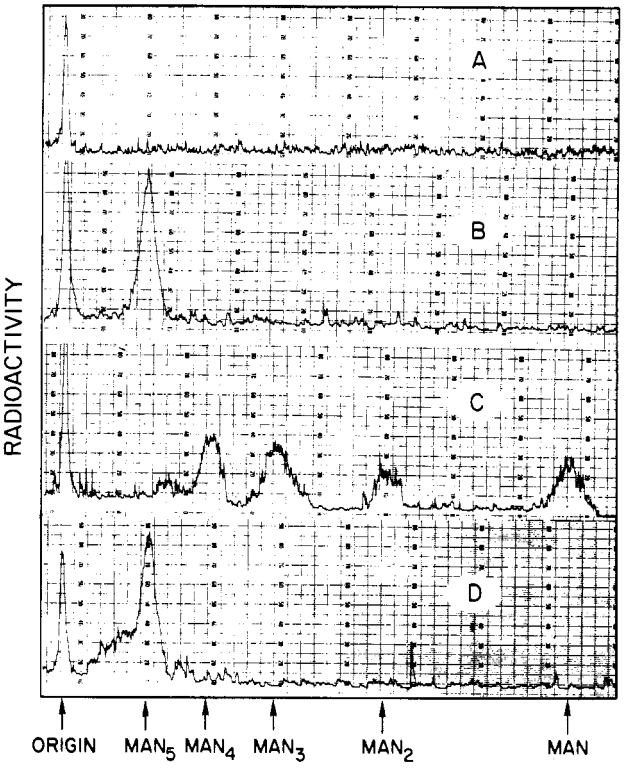

FIGURE 6: Partial acid hydrolysis products of Man_5P. The oligosaccharide was reduced with [^3H]$NaBH_4$ and subjected to partial acid hydrolysis, and the neutral or phosphatase-treated products were separated by paper chromatography. The positions of the reduced standards are shown at the bottom. A is the water effluent from a DEAE-Sephadex A-25 column to which the ^3H-reduced Man_5P was added, B is the phosphatase-treated material that was eluted from the column with 0.5 M NH_4HCO_3, C is the neutral water effluent from a DEAE-Sephadex A-25 column to which the acid-hydrolyzed Man_5P was added, and D is the phosphatase-treated material that was eluted from the same column with 0.5 M NH_4HCO_3. C shows that the largest neutral oligosaccharide was a tetrasaccharide, and D confirms that the smallest radioactive acidic oligosaccharide was the pentasaccharide. The radioactivity at the origin was added as a marker before the chromatogram was scanned.

Mannose 6-Phosphate Is at the Nonreducing End in Man_5P and Man_3P-B. Incubation of ^3H-rMan_5P and ^3H-rMan_3P (before separation into A and B fractions) with jack bean α-mannosidase did not shorten the chain length of either oligosaccharide, suggesting that phosphate blocked digestion because it was attached to the nonreducing terminal mannose. No β-anomeric linkage was detected in the ^1H NMR spectra of Man_5P or Man_3P-A + Man_3P-B.

For confirmation of the phosphate attachment, the products of partial acid hydrolysis were analyzed. ^3H-rMan_5P and ^3H-rMan_3P-B were partially hydrolyzed under mildly acidic conditions, the products were adsorbed to 1-mL columns of DEAE-Sephadex A-25, and neutral material was eluted with water and acidic material with 0.5 M ammonium bicarbonate. The neutral and acidic fractions, the latter after incubation with alkaline phosphatase, were analyzed by paper chromatography in solvent system B. Radiochromatogram scanning revealed that partial acid hydrolysis of ^3H-rMan_5P produced four radioactive neutral fragments that migrated with standard mannitol, mannobiitol, mannotriitol, and mannotetraitol. The acidic fraction after phosphatase treatment gave only one radioactive fragment that ran with standard mannopentaitol (Figure 6). Similar treatment of ^3H-rMan_3P-B gave a neutral fraction that consisted of radioactive mannitol and mannobiitol, and only a radioactive mannotriitol was detected in the acidic fraction after phosphatase treatment (Figure 7). These results

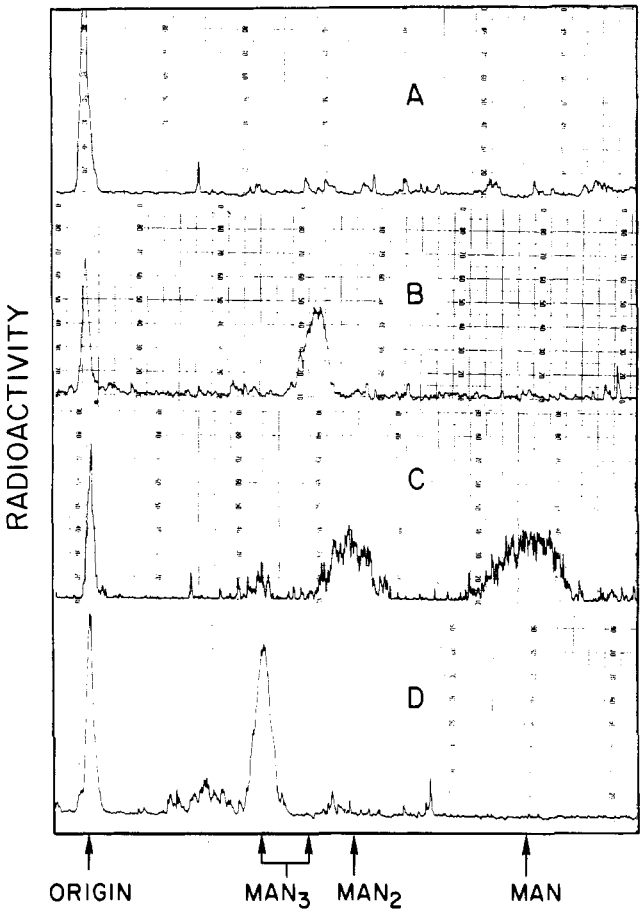

FIGURE 7: Partial acid hydrolysis of Man_3P-B. The phosphate monoester was reduced with [^{3}H]$NaBH_4$ and subjected to partial acid hydrolysis, and the neutral or phosphatase-treated products were separated by paper chromatography. The positions of the reduced standards are shown at the bottom, and because chromatograms B and D were developed for different times, the positions of the Man_3 peaks differ slightly. A is the water effluent from a DEAE-Sephadex A-25 column to which the ^{3}H-reduced Man_3P was added, B is the phosphatase-treated material that was eluted from the column with 0.5 M NH_4HCO_3, C is the neutral water effluent from a DEAE-Sephadex A-25 column to which the acid-hydrolyzed Man_3P was added, and D is the phosphatase-treated material that was eluted from the same column with 0.5 M NH_4HCO_3. C shows that the largest neutral oligosaccharide was a disaccharide, and D confirms that the smallest radioactive acidic oligosaccharide was the trisaccharide. The radioactivity at the origin was added as a marker before the chromatogram was scanned.

are consistent with phosphate attachment to the nonreducing-end mannose in both Man_5P and Man_3P-B. If phosphate were attached to any other mannose residue, the number and size of radioactive fragments, both neutral and acidic, would have been different from what we observed.

Linkage Analysis by ^{1}H NMR Spectroscopy. Acetolysis of Y-5 mannan produces five neutral oligosaccharides, mannose to mannopentaose, which Yen & Ballou (1974a) earlier characterized as having varying combinations of α1→2 and α1→3 linkages. Their methylation data suggested the presence of four isomers of the mannotriose, differing in the number and sequence of α1→2 and α1→3 linkages. Only one mannopentaose isomer was found, however; it contained three α1→2 linkages and one α1→3 linkage, and immunochemical evidence suggested that the α1→3 linkage was at the nonreducing end. Thus, in our linkage analyses of Man_5P and Man_3P-B, we were concerned with whether the Y-5 mannan was phosphorylated on one or more of the mannotriose isomers and whether the phosphorylated mannopentaose was different from the neutral mannopentaose observed previously.

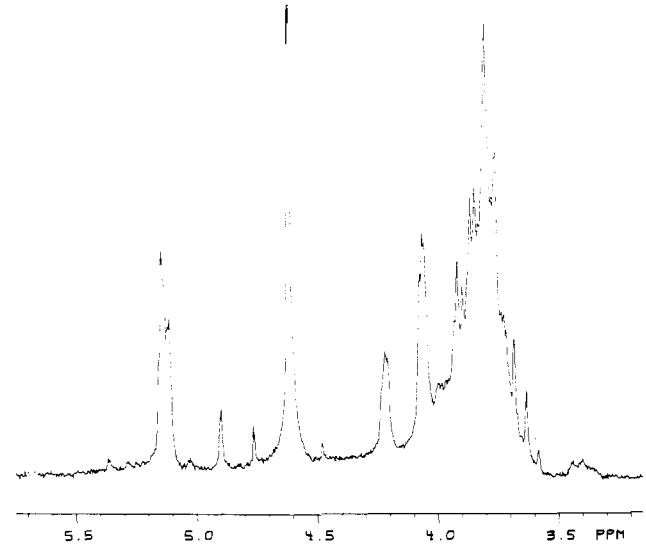

FIGURE 8: ^{1}H NMR spectrum of Man_3 from Man_3P-B. The two signals at ~5.15 ppm agree with the chemical shifts of a terminal nonreducing mannose, a terminal reducing mannose and an internal mannose all in α1→3 linkage. The signal at 4.9 ppm is the reducing mannose anomeric proton for the small fraction of the trisaccharide in the β configuration. Spinning side bands are present on each side of the HDO peak at 4.6 ppm. The signal at 4.22 ppm corresponds to the C-2 protons of the two 3-O-substituted mannoses.

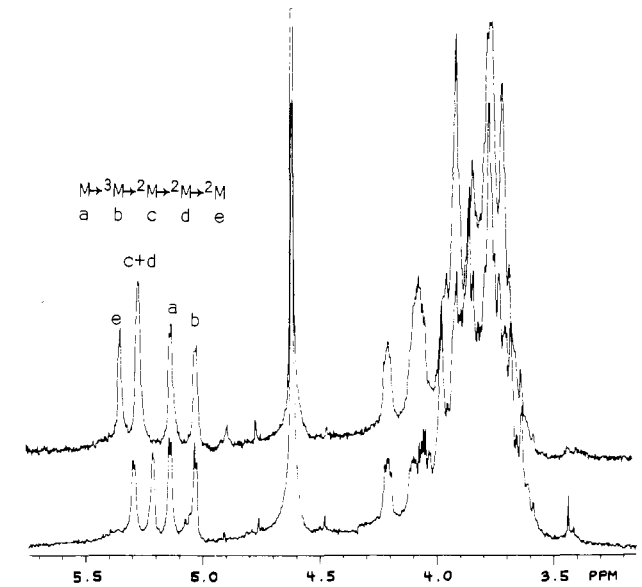

FIGURE 9: ^{1}H NMR spectra of Man_5 from Man_5P. The top spectrum is before and the bottom spectrum after reduction with $NaBH_4$. The insert indicates the assignments of the anomeric proton signals before reduction. After reduction, signal e is lost and signal d moves upfield to 5.22 ppm because this mannose unit is connected to a mannitol residue. Signal c remains at 5.3 ppm.

Man_3P-B was dephosphorylated with alkaline phosphatase and the product examined by ^{1}H NMR spectroscopy. The spectrum of Man_3 showed in the region typical of anomeric protons on α-mannopyranosides two partially overlapping signals, one twice the intensity of the other (Figure 8). By comparison with the anomeric proton chemical shifts of standard mannooligosaccharides (Cohen & Ballou, 1980), we ascribe these signals unambiguously to a mannotriose with the structure αMan1→3αMan1→3Man.

The linkage analysis of Man_5, obtained by dephosphorylation of Man_5P, was not as straightforward. Four anomeric proton signals equivalent to a total of five protons were observed in its spectrum (Figure 9). By comparison with

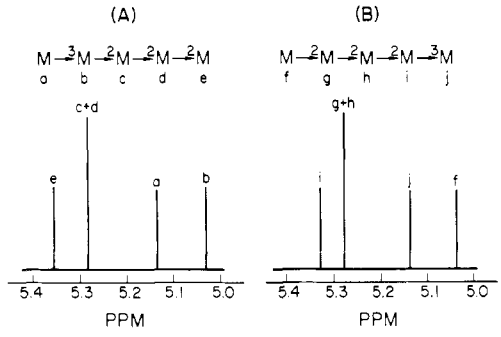

FIGURE 10: Reconstructed anomeric ^{1}H NMR spectra for two possible mannopentaose isomers. Reduction of compound A would be expected to eliminate signal e (the reducing end mannose) and lead to an upfield shift of signal d, whereas reduction of compound B should eliminate signal j and cause a shift of signal i. The results illustrated in Figure 9 support A for the structure of the mannopentaose from Man_5P.

standard mannooligosaccharides, we determined that two mannopentaose structures αMan1→3αMan1→2αMan1→2αMan1→2Man and αMan1→2αMan1→2αMan1→2αMan1→3Man could produce such a pattern of signals. The two isomers and their postulated anomeric proton spectra are illustrated schematically in Figure 10. The postulated signals of isomers A and B reveal a salient difference. The signal from the reducing-end mannose in isomer A would resonate furthest downfield of the four anomeric proton signals, whereas in B the reducing-end mannose signal is expected to appear farther upfield between two other signals. Thus, it seemed possible to distinguish between the two postulated structures of Man_5 by determining which of the signals disappeared after sodium borohydride reduction.

The spectrum of sodium borohydride reduced Man_5 showed four anomeric proton signals (Figure 9), and three of them were at the same frequency as before reduction whereas one signal moved to a new position. Most importantly, the signal furthest downfield in the spectrum of unreduced Man_5 disappeared after reduction. Thus, Man_5 has the structure αMan1→3αMan1→2αMan1→2αMan1→2Man, which is identical with the mannopentaose characterized previously (Yen & Ballou, 1974a). Support for this assignment came from the presence of the signal at 4.2 ppm in Figure 9 before and after reduction. This signal has been assigned to the C-2 proton of a mannose residue that is substituted at position 3 (Cohen & Ballou, 1980). In A, this signal would arise from the mannose next to the one at the nonreducing end, whereas in B, it would come from the reducing end mannose. Hence, if Man_5 had possessed structure B, the signal at 4.2 ppm should disappear because reduction would have converted the reducing-end mannose into mannitol. That the signal survived reduction supports the placement of the α1→3 linkage at the nonreducing end in Man_5.

Discussion

Partial acetolysis of *H. wingei* mannoprotein yields a series of neutral mannooligosaccharides from mono- to pentasaccharide (Yen & Ballou, 1974a). Our study shows that only two of these, a tri- and a pentamannoside, occur in the phosphorylated form. The neutral pentamannoside fragment obtained by acetolysis was shown previously (Yen & Ballou, 1974a) to have the structure αMan1→3αMan1→2αMan1→2αMan1→2Man, and the phosphorylated compound we have obtained is the analogous P→6αMan1→3αMan1→2αMan1→2αMan1→2Man. The composition of the compound, its ^{1}H NMR spectrum, and the spectrum of the reduced product are consistent only with the structure shown here. In addition, the ^{31}P NMR spectrum indicates that the phosphate is monoesterified, and the failure of jack bean α-mannosidase to act on the compound supports the conclusion that the phosphate group is attached to the mannose unit at the nonreducing end of the chain. The location of the phosphate on position 6 was demonstrated by a coupled enzyme assay for mannose 6-phosphate in the acid hydrolysate and by the splitting of the ^{31}P resonance into a triplet in the ^{1}H-coupled NMR spectrum.

The structure of the *H. wingei* pentasaccharide phosphate is similar to that of a compound produced by mild acid hydrolysis of the phosphomannan secreted by *Hansenula holstii* and characterized by Bretthauer et al. (1973) as P→6αMan1→3αMan1→3αMan1→3αMan1→2Man. In this instance, the product appears to result from degradation of a molecule like teichoic acid in which the pentasaccharide units are held together by phosphodiester bonds. This is a different kind of molecule from the mannoprotein we have isolated in which the pentasaccharide units are attached through the reducing end in 1→6 linkage to the polysaccharide component of the protein.

The trimannoside phosphate has a structure analogous to that of the pentasaccharide, with the phosphate group occupying position 6 of the nonreducing terminal mannose unit. In this instance, however, the three mannose units are connected by two α1→3 linkages. This compound differs from the trimannoside phosphate obtained by acetolysis of *S. cerevisiae* mannoprotein in that the latter has α1→2 linkages between the mannose units and the phosphate is linked to position 6 of the middle hexose unit (Thieme & Ballou, 1971; Rosenfeld & Ballou, 1974). It is also noteworthy that only one of the four isomeric mannotriose components of the *H. wingei* mannoprotein is phosphorylated.

Now that a catalogue of anomeric proton chemical shifts for α-linked mannooligosaccharides has been compiled (Cohen & Ballou, 1980), it is relatively easy to characterize such compounds. The ^{31}P NMR spectrum was also important in this work in revealing the presence of a trimannoside cyclic phosphate diester derivative in admixture with the phosphate monoester. Compounds of this type have not been noted previously in acetolysates of yeast mannoproteins. We assume it to be an artifact of the acetolysis reaction, but we were unable to confirm the presumed origin of the cyclic phosphate because mannose 6-phosphate did not yield a cyclic phosphodiester when subjected to acetolysis, although it does when treated with a carbodiimide reagent (Khorana et al., 1957). Although mannoproteins isolated without exposure to alkali show phosphodiester signals in the ^{31}P NMR spectra, owing to the presence of glycosyl phosphoryl diester bonds (Thieme & Ballou, 1971; Costello et al., 1975), the mannoprotein preparation we studied was isolated by Fehling's precipitation, and it clearly lacked phosphodiester linkages before acetolysis.

The role of phosphate in yeast cell wall mannoproteins is unknown, although it does provide a negative charge to the cell surface. Because mutants that lack phosphate in the cell wall function normally, the phosphate cannot be a critical component. It has also been noted that the phosphate, which makes up 5% of the weight of *H. wingei* 5-agglutinin, is not required for its activity in sexual agglutination: a mutant that makes the agglutinin without phosphate is able to mate, and the diploid sporulate, normally (Sing et al., 1976).

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