Tipper, D. J., and Strominger, J. L. (1965), *Proc. Nat. Acad. Sci. U. S. 54*, 1133.

Van Heijenoort, J., Elbaz, L., Dezélée, Ph., Petit, J.-F., Bricas, E., and Ghuysen, J.-M. (1969), *Biochemistry 8*, 207.

Warth, A. D., and Strominger, J. L. (1971), *Biochemistry 10*, 4349.

Wise, E. M., and Park, J. J. (1965), *Proc. Nat. Acad. Sci. U. S. 54*, 75.

Yamaguchi, T. (1965), *J. Bacteriol. 89*, 444.

Characterization of a Phosphorylated Pentasaccharide Isolated from *Hansenula holstii* NRRL Y-2448 Phosphomannan†

R. K. Bretthauer,* G. J. Kaczorowski,‡ and M. J. Weise‡

ABSTRACT: The phosphomannan secreted by the yeast *Hansenula holstii* NRRL Y-2448 has been degraded by mild acid hydrolysis of the hemiacetal phosphodiester linkages. This procedure yields: (1) a phosphorylated pentasaccharide which accounts for 65% of the carbohydrate in the intact polymer, (2) a high molecular weight phosphorylated core fragment which is resistant to further mild acid hydrolysis and which accounts for 9% of the carbohydrate in the intact polymer, and (3) other small fragments (tetrasaccharide and smaller) which have not been characterized. The structure of the phosphorylated pentasaccharide, determined by chemical, physical, and enzymatic methods, was concluded to be P-6-Man*p*-α-(1→3)-Man*p*-α-(1→3)-Man*p*-α-(1→3)-Man*p*-α-(1→2)-Man. No evidence for structural heterogeneity of this compound was apparent.

The phosphomannan produced exocellularly by the yeast *Hansenula holstii* NRRL Y-2448 has the constituents D-mannose and phosphorus in a molar ratio of 5:1 (Jeanes *et al.*, 1961). The phosphate is present in diester linkage between carbon-6 hydroxyl of one mannose unit and carbon-1 hemiacetal hydroxyl of another mannose unit (Slodki, 1962).

Jeanes and Watson (1962) proposed from periodate oxidation studies that the structure of this polymer may consist of a repeating unit of ten mannose units distributed on an average of five between a phosphodiester group linked between carbon-6 of one mannose unit and carbon-1 of another which is mannosidically linked at carbon-2. The remaining three mannosidic linkages in each pentamer unit were suggested to be 1→3′ in one of the mannopentaose units and two 1→3′ and one 1→2′ linkages in the other mannopentaose unit. That sequences of at least three 1→3′ linkages occur was demonstrated by isolation of the 1→3′-linked trisaccharide from periodate-oxidized phosphomannan Y-2448 (Jeanes *et al.*, 1962).

The questions as to whether this phosphomannan is comprised solely of such repeating monophosphomannopentaose units and whether these units are structurally homogeneous are of interest from the viewpoint of biosynthesis. Slodki (1962) suggested that these phosphomannans could be synthesized by appropriate transfers of mannosyl and mannose 1-phosphoryl units from GDP-mannose to the growing polysaccharide. We have since demonstrated both such transfer reactions from GDP-mannose with a particulate enzyme fraction from *H. holstii* (Bretthauer *et al.*, 1969; Kozak and Bretthauer, 1970), although it has not been established that the mannose 1-phosphoryl transfer was to exocellular phosphomannan. If a repeating unit structure exists in the phosphomannan, one may further suggest that this repeating unit is preassembled during biosynthesis on an intermediate acceptor molecule with subsequent polymerization of the repeating unit, analogous to the biosynthesis of bacterial peptidoglycan, the antigenic side chain of lipopolysaccharide, and capsular polysaccharide (for review, see Rothfield and Romeo, 1971).

This report presents evidence for the existence in phosphomannan Y-2448 of an apparently homogeneous monophosphomannopentaose unit which accounts for at least 65% of the mannose and phosphate in the native polymer. A core polysaccharide which lacks phosphodiester linkages accounts for about 10% of the remaining polymer.

Materials and Methods

Production of Phosphomannan Y-2448. H. holstii Y-2448 was grown at 25° in a medium containing 1 g of corn steep liquor, 1 g of tryptone, 5 g of KH_2PO_4, 5 ml of salts solution, and 40 g of glucose per l. (Anderson *et al.*, 1960). The shake flasks were inoculated with a 5% volume of a culture grown for 24 hr. After growth for 72–96 hr, the potassium salt of phosphomannan was isolated and purified as described by Jeanes *et al.* (1961). To obtain uniformly labeled [^{14}C]phosphomannan, 500 μCi of uniformly labeled [^{14}C]glucose was added to 2.5 ml of the above growth medium. This yielded, after 72-hr growth, 50 mg of phosphomannan having a specific radioactivity of 7×10^6 cpm/mg.

Hydrolysis of Phosphomannan. The solution contained

† From the Department of Chemistry, Program in Biochemistry and Biophysics, University of Notre Dame, Notre Dame, Indiana 46556. *Received October 25, 1972.* This work was supported by Research Grant AI-09062 from the National Institutes of Health and by a grant from Miles Laboratories, Inc.

‡ Participants in a National Science Foundation Undergraduate Research Program during tenure of this work. Present address: Department of Biology, Massachusetts Institute of Technology, Cambridge, Mass.

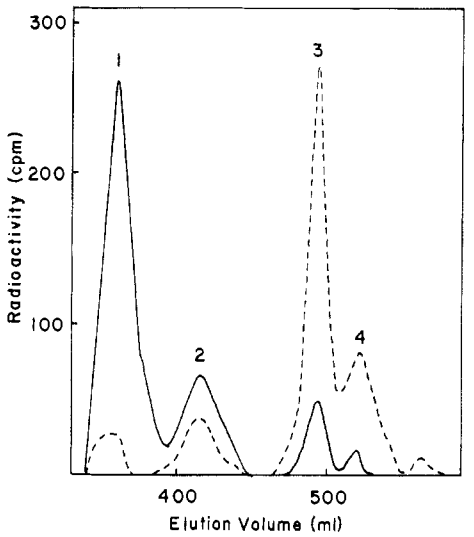

FIGURE 1: Typical elution patterns from Sephadex G-25 columns for hydrolyzed [^{14}C]phosphomannan. The phosphomannan was hydrolyzed at pH 2.54 as described in Materials and Methods. The gel column, equilibrated with 0.2 M NH_4HCO_3, was 230 × 2.2 cm. Fractions of 2 ml were collected at a flow rate of 10 ml/hr. The peak numbers refer to the fragments described in the text. (——) 0.5-hr hydrolysis; (– – –) 2-hr hydrolysis.

300 mg of phosphomannan and 0.5 g of KCl in 120-ml total volume with a final pH of 2.54. For the time study, this solution, containing [^{14}C]phosphomannan (7.7 × 10^5 cpm/mg), was divided into 20-ml aliquots. After hydrolysis for different periods of time at 100°, the solutions were cooled, adjusted to pH 6.9 with NH_4HCO_3, and evaporated to dryness several times from water at 40° on a rotary flask evaporator.

Enzymatic hydrolysis was carried out with partially purified *Arthrobacter* α-mannosidase as described by Jones and Ballou (1969). The organism was kindly supplied by Dr. C. E. Ballou.

Isolation of Phosphorylated Pentasaccharide. A solution containing 5.4 g of phosphomannan and 2 g of KCl in 200 ml at pH 2.42 was hydrolyzed 4 hr at 100°. After cooling and neutralization to pH 6.9, a small amount of insoluble residue was removed by centrifugation. Barium acetate (2 g) was dissolved in the supernatant liquid which was then made basic to a phenolphthalein end point with NaOH. Addition of 20 ml of ethanol precipitated any unhydrolyzed phosphomannan and core material which was removed by centrifugation. An additional two volumes (400 ml) of ethanol were added which precipitated the barium salt of the pentasaccharide phosphate. The precipitate was dissolved in water and again submitted to ethanol fractionation. The final product was dissolved in water by mild acidification, decationized with Dowex 50 (H^+) resin, and neutralized with NH_4OH. The yield was 1.5 g after lyophilization.

Preparation of Standard Oligosaccharides. Bakers yeast mannan was isolated and submitted to acetolysis as described by Stewart *et al.* (1968). The tetrasaccharide obtained from gel filtration of the deacetylated acetolysis products was then methylated and methanolyzed. The retention times on gas–liquid chromatography of the products and their trimethylsilyl derivatives were nearly identical with those reported by Stewart *et al.* (1968) for methyl 2,3,4,6-tetra-*O*-methylmannoside, methyl 3,4,6-tri-*O*-methylmannoside, and methyl 2,4,6-tri-*O*-methylmannoside. These products were present in a ratio of 1:2:1, respectively.

Methylation and Trimethylsilylation Procedures. Carbohydrates were methylated with silver oxide–methyl iodide as described by Stewart *et al.* (1968). After methanolysis, care was taken in the evaporation steps to avoid losses of methylated sugars. Trimethylsilyl derivatives were prepared by the method of Sweeley *et al.* (1963). After addition of reagents to the sample, the reaction vessel was placed on a shaker for one hour before examination by gas chromatography.

Radioactivity Measurements. Aqueous samples were counted in a liquid scintillation counter using Bray's solution (0.1-ml sample in 10 ml of cocktail) or a Triton X-100–toluene (2:1 v/v) cocktail (0.6-ml sample in 8 ml of cocktail). In both cases, counting efficiency was approximately 70%. Paper chromatogram strips were counted in 10 ml of toluene cocktail with a counting efficiency of approximately 80%.

Analytical Methods. Total carbohydrate was determined by the phenol–H_2SO_4 method (Dubois *et al.*, 1956) using mannose as a standard, and phosphate was measured by the method of Ames and Dubin (1960).

Chromatographic Techniques. Descending paper chromatography was carried out on S+S 589 Green Ribbon Paper. Thin-layer plates of approximately 0.25 mm thickness were prepared with silica gel G. The following solvents were used (in volume ratios): (A) 1-butanol–pyridine–water (6:4:3), (B) methyl ethyl ketone–boric acid saturated water–glacial acetic acid (9:1:1), (C) ethanol–methyl ethyl ketone–0.5 M morpholinium tetraborate (pH 8.6) in 0.01 M EDTA (7:2:3), (D) 95% ethanol–1 M ammonium acetate (pH 7.5) (7:3), and (E) benzene–methanol (93:7).

Sugars were detected on paper chromatograms with periodate–benzidine (Gordon *et al.*, 1956) or alkaline silver nitrate (Trevelyan *et al.*, 1950). Methylated sugars on thin-layer plates were detected by sulfuric acid charring.

Gas–liquid chromatography was carried out on a Hewlett Packard 5750B chromatograph equipped with a hydrogen flame ionization detector and a 6 ft × 1/8 in. stainless steel column packed with 10% Carbowax 20M on Chromosorb W. A column temperature of 195° was employed for analysis of methylated sugars and a temperature of 175° for trimethylsilyl derivatives. The injection port temperature was 220°. Nitrogen was employed as the carrier gas at a flow rate of 20 cm^3/min. Peak areas were obtained with a K+E polar planimeter.

Proton Magnetic Resonance. Phosphorylated pentasaccharide was reduced with $NaBH_4$ and after acidification, the boric acid was removed by repeated evaporation from methanol. The sample was evaporated to dryness five times from D_2O and finally dissolved in D_2O at a concentration of 10%. The proton magnetic resonance spectra were recorded at 30° on a Varian XL-100 spectrometer. Chemical shifts are expressed relative to the trimethylsilyl line of sodium 2,2-dimethyl-2-silapentane-5-sulfonate used as an internal standard.

Other Materials. Bacterial alkaline phosphatase (Type III) was purchased from Sigma, uniformly labeled [^{14}C]glucose and sodium [^{3}H]borohydride from New England Nuclear, DEAE-cellulose from Bio-Rad, and Sephadex (Fine grades) from Pharmacia.

Results

Time-Dependent Hydrolysis of Phosphomannan. [^{14}C]Phosphomannan was hydrolyzed at pH 2.54 as described in

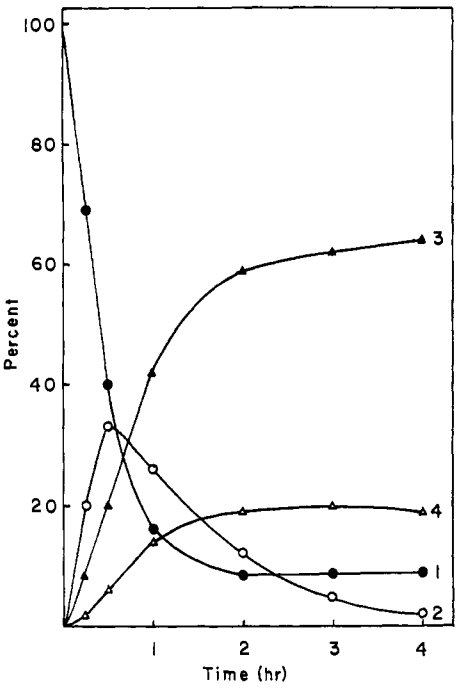

FIGURE 2: Acid degradation profile for phosphomannan. The areas of the peaks from Sephadex G-25 gel filtrations (such as in Figure 1) were determined and the per cent of each component present at a given time of hydrolysis was calculated. The numbers to the right of each curve refer to the components shown in Figure 1 and to the fragments described in the text.

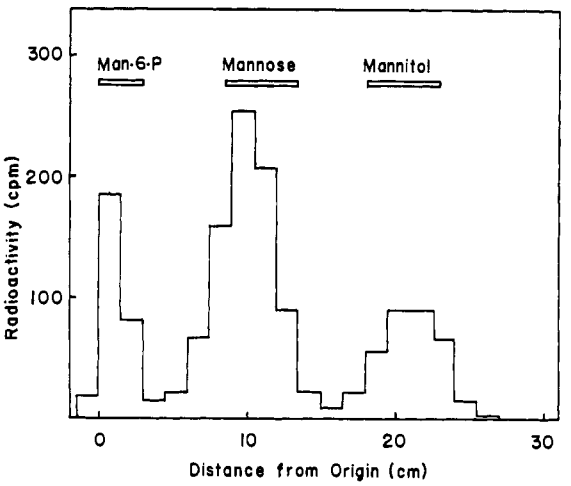

FIGURE 3: Radiochromatogram of reduced, acid-hydrolyzed ^{14}C-labeled fragment 3. Approximately 1 mg of purified ^{14}C-labeled fragment 3 in 0.1 ml of 0.1 N NaOH was reduced with excess $NaBH_4$ for 1.5 hr at 50°. The desalted solution was hydrolyzed in 1 N HCl at 100° for 3 hr. Chromatography was carried out with solvent B for 6 hr. The paper was cut into 1.5-cm strips for liquid scintillation counting.

Materials and Methods for periods of 0.25, 0.5, 1, 2, 3, and 4 hr. The samples were fractionated by chromatography on Sephadex G-25 columns into four major fragments. Typical elution profiles are shown in Figure 1 for the 0.5- and 2-hr samples. It was evident that hydrolysis for increasing lengths of time produced at least three fragments (numbered 2, 3, and 4 in Figure 1) which did not vary in elution volume. Fragment 1 is eluted near the void volume and therefore conclusions regarding its molecular size are not appropriate.

The peak areas were measured for each of the four fragments and compared to the total area. The degradation profile obtained from plotting these fractional areas *vs.* time (Figure 2) shows that fragment 3 comprises 42% of the total sample carbohydrate after 1-hr hydrolysis, 59% after 2 hr, and then only slowly increases to 64% after 4 hr. Also evident is that fragment 2 is an intermediate species which accumulates rapidly but then is further degraded to fragment 3 or to fragments 3 and 4. Fragment 4 accounts for 19% of the total carbohydrate after 2-hr hydrolysis and does not change appreciably in concentration with longer periods of hydrolysis. The 9% of the polymer (fragment 1) that was not ultimately degraded to fragments 3 and 4 eluted at the void volume of Sephadex G-25 and Sephadex G-50 and presumably represents an acid-resistant core structure of the phosphomannan.

Composition of Hydrolysis Products. FRAGMENT 3. This fragment, which will be shown to be a phosphorylated pentasaccharide, was prepared in large quantities by ethanol fractionation of the barium salt from 4-hr hydrolysates as described in Materials and Methods. The elution position from Sephadex G-25 was identical with that as shown for fragment 3 in Figure 1.

Chromatography on Sephadex G-15 (150 × 1.5 cm, 0.1 M NH_4HCO_3) revealed a single peak which, other than for approximately 10% of the leading edge, had a constant mannose:phosphate ratio of 5:1. These latter fractions were pooled and chromatographed on DEAE-cellulose (30 × 2 cm, linear gradient of 0.01–0.5 M NH_4HCO_3). The sample eluted as a single component at 0.13 M NH_4HCO_3 with a mannose:phosphate ratio of 5:1. Chromatography on Bio-Gel P-2 (175 × 2.3 cm, 0.2 M NH_4HCO_3) now gave a single, symmetrical peak with a constant mannose:phosphate ratio. Homogeneity was further indicated by paper chromatography using solvents A, B, C, and D and by paper electrophoresis (0.05 M sodium tetraborate, pH 8.7). The yield after the three column chromatographic steps was 75%.

^{14}C-labeled fragment 3, prepared as described in the preceding paragraphs, was reduced with $NaBH_4$. The solution, after treatment with Dowex 50 (H^+) resin, was dried on a rotary evaporator and the drying procedure was repeated three times from methanol. The residue was acid hydrolyzed (1 N HCl, 100°, 3 hr) and dried in a vacuum desiccator at 4°. Chromatography of the products in solvent B followed by liquid scintillation counting of the paper strips gave three radioactive components which migrated with standard mannose-6-P, mannose, and mannitol, and which were present in an isotope ratio of 0.98:2.7:1.0, respectively (Figure 3). This is close to the expected 1:3:1 ratio for a pentasaccharide phosphate.

The glycosidic linkage to the terminal reducing mannose unit was shown to be 1→2′ in the following manner. ^{14}C-labeled fragment 3 was reduced with sodium borotritide (1.87×10^6 cpm/mg) and the reduced fragment then freed of salts by Sephadex G-15 chromatography. Oxidation with 0.05 M $NaIO_4$ for 22 hr in the dark at room temperature was followed by the addition of excess ethylene glycol and evaporation to dryness. Reduction was again carried out with unlabeled $NaBH_4$, the salts were removed as above, and the sample was then acid hydrolyzed (1 N HCl, 100°, 3.5 hr). The solution was evaporated at 4° in a partially evacuated desiccator containing NaOH pellets. Composition of the neutral products in this residue was examined by chromatog-

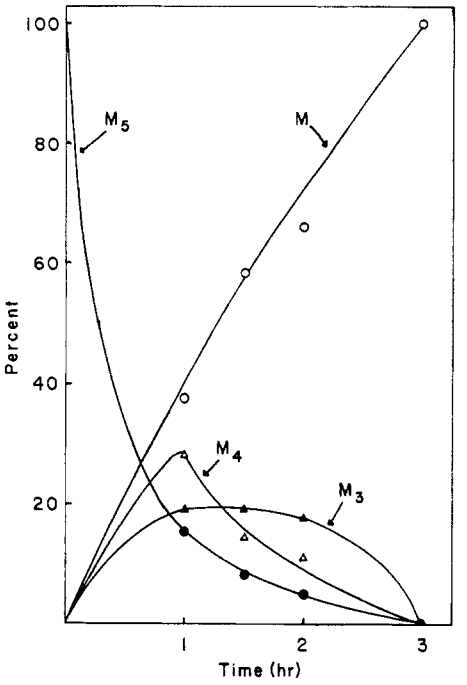

FIGURE 4: Degradation profile for the [^{14}C]oligosaccharide (dephosphorylated ^{14}C-labeled fragment 3) upon exposure to α-mannosidase. [^{14}C]Oligosaccharide (3×10^4 cpm) was treated with 20 units of α-mannosidase in 0.5-ml total volume at 25°. Aliquots were removed at 0, 1, 1.5, 2, and 3 hr and submitted to paper chromatography with solvent A for 15 hr. Radioactivity was determined by liquid scintillation counting of 1-cm wide paper strips. Results are expressed as the per cent of total radioactivity found in each component. M, M_3, M_4, and M_5 refer to mannose and oligomers thereof.

raphy in solvent B which separates mannose, erythritol, and glycerol. The paper strips were counted in the liquid scintillation counter for their ^{3}H and ^{14}C contents. Glycerol, containing both ^{3}H and ^{14}C, was present which indicates a 1→2′ linkage to the reducing mannose unit. The only other neutral component migrated as [^{14}C]mannose and was present in an isotope ratio to [^{14}C]glycerol of 4.8:1. Assuming that the carbon atoms in the mannose residues are equally labeled with ^{14}C and that the other three glycosidic linkages are 1→3′, the theoretical ratio is 6:1.

DEPHOSPHORYLATED FRAGMENT 3. To 75 mg of sample in 5 ml of 0.2 M NH_4HCO_3 was added 500 μg of alkaline phosphatase and a drop of toluene. After 12 and 24 hr at 25°, additional alkaline phosphatase was added. At 36 hr, when 80% of the sample phosphate was present as inorganic phosphate, the solution was evaporated to dryness several times to remove the NH_4HCO_3. The residue was suspended in 2 ml of water and, after centrifugation, the clear supernatant fluid was applied to a DEAE-Sephadex A-25 column (35 × 1.5 cm) equilibrated with 0.01 M NH_4HCO_3. The neutral oligosaccharide was eluted directly in a symmetrical peak with 0.01 M NH_4HCO_3. Neutral [^{14}C]oligosaccharide prepared in the same way gave a 3.96:1 ratio of mannose:mannitol after $NaBH_4$ reduction, acid hydrolysis, and paper chromatography in solvent B, thus confirming that the oligosaccharide was a pentamer. The unlabeled oligosaccharide was methylated as described in Materials and Methods and the methanolysis products were examined by gas chromatography. As shown in Table I, the methyl glycosides of 2,3,4,6-tetra-*O*-methyl-, 3,4,6-tri-*O*-methyl-, and 2,4,6-tri-*O*-methylmannose were detected in a ratio of 1.00:0.98:3.14, respectively. No di-*O*-methyl derivatives were detected. As the 2,3,4- and 3,4,6-tri-*O*-methyl derivatives are not separated on this column, the methyl ethers were further analyzed by thin-layer chromatography and as their trimethylsilyl derivatives by gas chromatography. No methyl 2,3,4-tri-*O*-methylmannoside was detected by either method (Table I), thus eliminating the possibility of any 1→6′ mannosidic linkages occurring in the pentasaccharide.

TABLE 1: Gas–Liquid and Thin-Layer Chromatography of Methylated Oligosaccharides.

Mannose Derivative		Retention Time[a]		Area		R_F Values[a]	
-*O*-Methyl	-*O*-Me$_3$Si	M_4[e]	M_5	M_4[e]	M_5	Lit.[c]	M_5
1,2,3,4,6		1.00	1.00	1.00[d]	1.00[d]	1.00	1.00
1,3,4,6		2.04	2.03	2.05	0.98	0.34	0.32
1,2,3,4		(2.03)[b]				0.25	
1,2,4,6		2.34	2.35	0.95	3.14	0.46	0.52
1,3,4,6	2	0.47	0.48				
1,2,3,4	6	(0.66)[b]					
1,2,4,6	3	0.72	0.73				

[a] Relative to methyl 2,3,4,6-tetra-*O*-methylmannoside. [b] Not detected in these studies; retention time from Stewart *et al.* (1968). [c] From Stewart *et al.* (1968). [d] Includes 5% of β anomer with retention time of 1.29. [e] Mannotetraose from bakers yeast (Lee and Ballou (1965)).

The anomeric configuration of the glycosidic linkages in the pentasaccharide phosphate was determined by both enzymatic and nuclear magnetic resonance (nmr) techniques. For the enzymatic study, ^{14}C-labeled oligosaccharide (dephosphorylated) was treated with *Arthrobacter* GJM1 α-mannosidase for different periods of time and the radioactivity in the hydrolysis products measured after separation by paper chromatography. Complete enzymatic hydrolysis to [^{14}C]-mannose occurred in 3 hr (Figure 4). At earlier time periods the tri- and tetrasaccharides were present, but at no time was disaccharide detectable. Any dimer formed, which would have a 1→2′ mannosidic linkage, is presumably very rapidly hydrolyzed as compared to the rate of hydrolysis of 1→3′ linkages. Similar rate differences for hydrolysis of 1→2′- and 1→3′-mannosidic linkages were observed previously by Jones and Ballou (1969).

The proton magnetic resonance spectrum was also consistent with the anomeric linkages being of the α configuration. Phosphorylated pentasaccharide was first reduced with $NaBH_4$ to eliminate signals from the anomeric hydrogens on the reducing end (Whyte, 1971). Examination of the reduced oligosaccharide revealed signals in the region characteristic of anomeric hydrogen absorption, at τ 4.91 and 5.02 in an approximate area ratio of 2.6:1 (Figure 5). The positions of these signals agree closely to those reported by Lee and Ballou (1965) for oligosaccharides from yeast cell wall mannan which contain only α linkages. The ratio of peak intensities for the observed signals also gives further evidence that the oligosaccharide is composed of five mannose units. This ratio should theoretically be 3:1 if the three hydrogens at the 1→3′ linkages resonate at the same frequency and the hydrogen at the single 1→2′ linkage resonates at a different frequency.

FRAGMENT 2. The structure of this compound has not been studied extensively, but appears to be a dimer of fragment 3. It migrates as a single component in paper chromatography with solvents A and D. A molecular weight of approximately twice that of fragment 3 is obtained from Sephadex G-25 gel filtration. Treatment with alkaline phosphatase releases 50% of the total phosphate, indicating that a phosphomonoester and a phosphodiester linkage is present. Finally, further mild acid hydrolysis converts this fragment into fragment 3 (also suggested in Figure 2).

FRAGMENT 1. Preliminary studies were carried out on material which was isolated as the barium salt from a 4-hr hydrolysate. The material elutes at the void volume of Sephadex G-50 and is thus still of relatively high molecular weight. Chromatography on DEAE-cellulose (Cl) with a LiCl gradient gives a single, symmetrical peak in which the phosphorus and carbohydrate elute simultaneously. The mannose:phosphate ratio is 7:1. After treatment of fragment 1 with Dowex 50 (H^+) resin, titration with NaOH revealed that the phosphate was predominantly phosphomonoester (inflection points at pH 4.7 and 8.8). However, extensive treatment with alkaline phosphatase resulted in only 25% of the phosphate being released as inorganic phosphate. The reason for this is not known. Preliminary methylation studies suggest that, in addition to 1→2′ and 1→3′ linkages, 1→6′ linkages are present. Because of the necessity of methylating the phosphorylated fragment, there latter results are only suggestive.

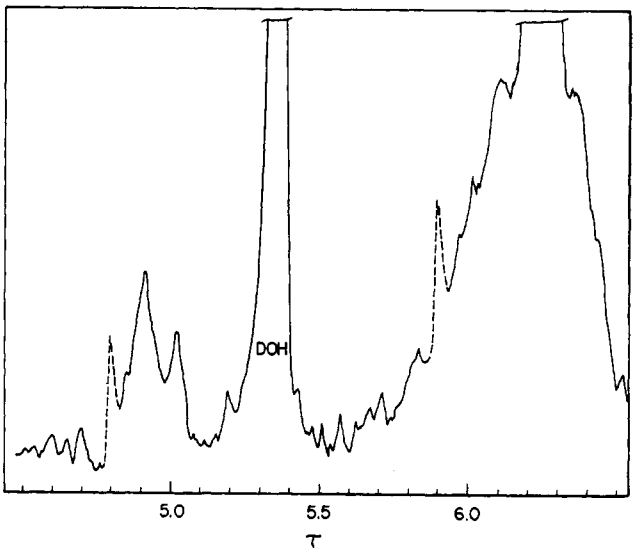

FIGURE 5: Proton magnetic resonance spectrum of reduced, phosphorylated oligosaccharide. The two signals represented by the dotted lines at τ 4.8 and 5.9 are spinning side bands and do not mask other proton signals.

Discussion

The purpose of this investigation was to examine in more detail the structure of phosphomannan Y-2448 and search for a possible repeating unit structure. The evidence presented indicates that at least 65% of the polymer carbohydrate and phosphate is present as a phosphorylated pentasaccharide (fragment 3). No heterogeneity was evident by the various chromatographic techniques applied to the phosphorylated and dephosphorylated oligosaccharide. Results from methylation indicated the presence of one 1→2′- and three 1→3′-mannosidic linkages. This information, coupled with the demonstration of the 1→2′ linkage occurring at the reducing end, is fully consistent with the structure P-6-Man*p*-α-(1→3)-Man*p*-α-(1→3)-Man*p*-α-(1→3)-Man*p*-α-(1→2)-Man. The α configuration of the mannosidic linkages is evident from the complete hydrolysis of the dephosphorylated oligosaccharide with α-mannosidase. Although oligosaccharides of mannose containing β-glycosidic linkages were not available for comparison in the proton magnetic resonance studies, the signals observed from the phosphorylated pentasaccharide in the region characteristic of anomeric hydrogens were at a position expected for α-mannosidic linkages and thus confirms the α-mannosidase results.

The mannose:glycerol ^{14}C ratio obtained after Smith degradation of the phosphorylated [^{14}C]pentasaccharide indicates that the phosphate is esterified to the terminal nonreducing mannose unit. With the phosphate in this position, the three internal mannose units are not oxidized because of substitution at carbon-3 and thus are recovered intact to give a mannose:glycerol isotope ratio (4.8:1) which most nearly approximates the theoretical 6:1 ratio. The possible presence of the phosphate on any of the mannose units other than the terminal nonreducing unit can be eliminated also by the following considerations. First, if phosphate were esterified to the reducing mannose unit (which could arise from the polymer having a [-man*p*→1-P-6→]$_n$ backbone with tetrasaccharide branches at carbon-2), mannitol-6-P would have been formed after reduction and hydrolysis. This has not been observed. Second, if phosphate were esterified to any one of the three internal mannose residues which are also substituted at carbon-3, the Smith degradation would have yielded a mannose:glycerol ^{14}C ratio of 2:1 (recovery of two intact mannose units and an additional glycerol from the terminal nonreducing mannose unit). Also, the alkaline β elimination of the phosphate as inorganic phosphate after periodate oxidation of the intact polymer could not occur (Jeanes and Watson, 1962). All of these observations are thus consistent with the phosphate being esterified to the terminal nonreducing mannose unit.

The yields of the methylated mannose units from the methylated, dephosphorylated pentasaccharide argues against linkage heterogeneity in the sample as isolated. Additional information on this point can be obtained from the periodate–$NaBH_4$ studies on the phosphorylated [^{14}C]oligosaccharide, for if additional 1→2′ linkages were present, the mannose:glycerol ^{14}C ratios would differ. Thus, if there were one 1→2′ and three 1→3′ linkages, this ratio would be 6:1; if two 1→2′ and two 1→3′ linkages, the ratio would be 2:1; if an equal mixture of the two previous structures, the ratio would be 3.3:1. The observed ratio of 4.8:1 most nearly approximates the expected value for a homogeneous sample containing one 1→2′ and three 1→3′ linkages. This does not agree with the structure proposed by Jeanes and Watson (1962), but it is probable that the additional 1→2′ linkages, as indicated by the periodate oxidation studies of the latter authors, are present either in the acid-resistant core of the polymer (fragment 1) or in fragment 4 of Figure 2. Further studies are clearly needed to verify this point.

The occurrence of a high molecular weight, acid-resistant fraction (fragment 1) in this phosphomannan has been previously observed (Slodki *et al.*, 1972; Dr. M. E. Slodki, personal communication). The phosphate in this core fraction is monoesterified as shown by titration. The apparent lack of phosphomonoesters in the intact polymer then suggests that phosphorylated pentasaccharide or polymerized units of it (fragment 2 or larger) may be esterified through the hemi-

acetal hydroxyl of the reducing end to the core phosphate to form a phosphodiester linkage branch point. The nonreducing termini of this proposed polymer structure would then be nonphosphorylated pentasaccharide units, and depending on the degree of branching in the polymer, may be present in quite significant amounts. Fragment 4 of Figure 1 may represent or contain such terminal oligosaccharide units as very little phosphate is present in this fraction. A more detailed structural analysis of this fragment is currently being carried out.

Our preliminary observation of 1→6′ linkages in the core fragment is of interest because of the common occurrence of a 1→6′-linked backbone in yeast cell wall mannans (Stewart and Ballou, 1968; Kocourek and Ballou, 1969). Whether any biosynthetic similarities or precursor–product relationship exists between the exocellular phosphomannans and cell wall mannans remains to be determined.

References

Ames, B. N., and Dubin, D. T. (1960), *J. Biol. Chem. 235*, 769.

Anderson, R. F., Cadmus, M. C., Benedict, R. G., and Slodki, M. E. (1960), *Arch. Biochem. Biophys. 89*, 289.

Bretthauer, R. K., Kozak, L. P., and Irwin, W. E. (1969), *Biochem. Biophys. Res. Commun. 37*, 820.

Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A., and Smith, F. (1956), *Anal. Chem. 28*, 350.

Gordon, H. T., Thornburg, W., and Werum, L. H. (1956), *Anal. Chem. 28*, 848.

Jeanes, A., Pittsley, J. E., Watson, P. R., and Dimler, R. J. (1961), *Arch. Biochem. Biophys. 92*, 343.

Jeanes, A., Pittsley, J. E., Watson, P. R., and Sloneker, J. H. (1962), *Can. J. Chem. 40*, 2256.

Jeanes, A., and Watson, P. R. (1962), *Can. J. Chem. 40*, 1318.

Jones, G. H., and Ballou, C. E. (1969), *J. Biol. Chem. 244*, 1043.

Kocourek, J., and Ballou, C. E. (1969), *J. Bacteriol. 100*, 1175.

Kozak, L. P., and Bretthauer, R. K. (1970), *Biochemistry 9*, 1115.

Lee, Y.-C., and Ballou, C. E. (1965), *Biochemistry 4*, 257.

Rothfield, L., and Romeo, D. (1971), *Bacteriol. Rev. 35*, 14.

Slodki, M. E. (1962), *Biochim. Biophys. Acta 57*, 525.

Slodki, M. E., Ward, R. M., and Cadmus, M. C. (1972), *Develop. Ind. Microbiol. 13*, 428.

Stewart, T. S., and Ballou, C. E. (1968), *Biochemistry 7*, 1855.

Stewart, T. S., Mendershausen, P. B., and Ballou, C. E. (1968), *Biochemistry 7*, 1843.

Sweeley, C. C., Bentley, R., Maketa, M., and Wells, W. W. (1963), *J. Amer. Chem. Soc. 85*, 2497.

Trevelyan, W. E., Proctor, D. P., and Harrison, J. S. (1950), *Nature (London) 166*, 444.

Whyte, J. N. C. (1971), *Anal. Biochem. 42*, 476.

Model Studies on the Effects of Neutral Salts on the Conformational Stability of Biological Macromolecules. I. Ion Binding to Polyacrylamide and Polystyrene Columns†

Peter H. von Hippel,* Virginia Peticolas, Lise Schack, and Lynne Karlson

ABSTRACT: Measurements are reported of the binding constants to polyacrylamide gels of various neutral salts that can serve as perturbants of the stability of macromolecular conformations. Recycling chromatographic methods are used to obtain accurate values of these small numbers. It is shown by comparative measurements on polystyrene columns that binding occurs only to the amide moieties, and that ions are neither selectively attracted into, nor excluded from, the hydration shell surrounding nonpolar groups. Binding constants ($K_{a,rel}$) are defined *relative* to the binding of a tritiated water tracer, those measured for macromolecular conformation destabilizing ions showing positive values of $K_{a,rel}$, and those measured for conformation-stabilizing ions yielding negative $K_{a,rel}$ values. Single-ion binding constants, and values of ΔH° and ΔS° for the binding process, are also defined and determined. It is shown that the acrylamide moiety has a polar/nonpolar ratio comparable to the average group exposed in a protein as a consequence of a thermally induced unfolding process, and values are calculated for the free energy of transfer (per acrylamide unit) from water to various stabilizing or denaturing salts. These values can then be used to calculate the change in free energy stabilizing the native conformation of a protein which accompanies the transfer of the macromolecule into a conformation-perturbing solvent of given composition, when the change in peptide group exposure on unfolding is known. Alternatively, using melting temperature depression data, these values can be used to calculate the net number of amide groups exposed in transitions for which the molecular details are not known.

A considerable body of research over the past 10 or more years has established beyond doubt that concentrated solutions of various neutral salts have profound effects on the conformational stability of a variety of biological macromolecules and macromolecular assemblies. In essence, the effect may be viewed as a shifting, by the added salt, of the transition boundary between an ordered macromolecular structure (in which residue–residue contacts are thermodynamically favored for at least the groups comprising the "interior" of

† From the Institute of Molecular Biology and the Department of Chemistry, University of Oregon, Eugene, Oregon 97403. *Received September 27, 1972.* This research was supported by U. S. Public Health Service Research Grant No. GM-15792 (to P. H. v. H.).