Nature of the Phosphodiester Linkage of the Phosphomannan from the Yeast *Kloeckera brevis**

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ABSTRACT: The cell wall phosphomannan of *Kloeckera brevis* has been extracted and separated by ion exchange into four fractions with mannose to phosphate ratios varying from 20 to 6.5. All of the phosphate was diesterified, as shown by titration. Acid hydrolysis of the phosphodiester mannan at pH 2.1 at 100° released mannose with a half-life of 3.3 min in an amount equivalent to the phosphate content. In the intact mannan, this mannose was esterified to phosphate through position 1 in α configuration as shown by proton magnetic resonance studies. Smith degradation, partial acid hydrolysis, and ^{31}P nuclear magnetic resonance demonstrated that the phosphate, in turn, was esterified to position 6 of the middle mannose of a trisaccharide side-chain unit obtained by acetolysis of the phosphomannan. Phosphate was present in a similar structure in *Saccharomyces cerevisiae* phosphomannan, except that mannobiose as well as mannose was released by mild acid hydrolysis; and the phosphate, in turn, was esterified to tri- and tetrasaccharide side-chain units. These structures are similar to those previously found in other types of phosphomannans.

Polymers of D-mannose comprise 30–50% of the yeast cell wall (McMurrough and Rose, 1967). Some of these mannans are monodisperse, with molecular weights ranging from 22,000 to 330,000, depending on the yeast and method of isolation (Sentandreu and Northcote, 1968; Jones and Ballou, 1969; Eddy and Longton, 1969). The structure of many yeast mannans is based on a backbone of α-1→6-linked D-mannose units substituted by side chains of one to six α-1→2- and α-1→3-linked D-mannose units (Stewart *et al.*, 1968; Jones and Ballou, 1969). The length and proportion of side chains varies greatly between species (Kocourek and Ballou, 1969), and β as well as α linkages have been reported for some yeast mannans (Gorin *et al.*, 1969).

In addition to the carbohydrate structure outlined above, yeast mannans contain from 1 to 6% protein (Sentandreu and Northcote, 1958; Gorin *et al.*, 1971), from 0.12 to 8.5% *N*-acetylglucosamine (Gorin *et al.*, 1971), and from 0.04 to 8.5% phosphate. Mill (1966) found the phosphate to be diesterified and isolated mannose 6-phosphate from hydrolysates of *Saccharomyces cerevisiae* mannan. Stewart and Ballou (1968) found diesterified phosphate in *Kloeckera brevis* mannan, but concluded that the phosphate was attached to position 3 or 4 of a mannose in the backbone.

In addition to the physical protection mannans must provide for the yeast cell, they have been implicated in such phenomena as anchoring of enzymes to the cell wall (Gascon *et al.*, 1968) and in cell budding (Nickerson and Falcon, 1956). We recently proposed (Thieme and Ballou, 1970) that yeast cell wall mannan may be involved in cell surface recognition between yeast cells of opposite mating types. Although it was concluded that there is no gross difference in strucutre between mannans from haploid yeasts of opposite mating type, the minor components of the mannan may still play a role in such cell interaction.

In this paper we report the complete structure of the phosphodiester component in *K. brevis* yeast cell wall mannan and present some preliminary conclusions on the structure of the phosphate ester of *S. cerevisiae* mannan. In both cases, the phosphate is shown to be present as α-D-mannosyl 1-phosphate units attached in diester linkage to position 6 of mannose units in the sidechains of the mannans rather than to mannose units in the backbone, as previously suggested (Stewart and Ballou, 1968).

Experimental Section

Materials. K. brevis (H. Phaff 55-45) and *S. cerevisiae* (238C) were grown as previously described (Stewart and Ballou, 1968). The harvested cells were autoclaved for 90 min in 0.2 M citrate buffer (pH 7.0). After centrifugation, carbohydrate was isolated from the supernatant fluid by ethanol precipitation and treatment with 1 M acetic acid, as described by Stewart and Ballou (1968).

Alkaline phosphatase (BAPC) was obtained from Worthington Biochemical Corp. α-Mannanase was purified from *Arthrobacter* GJM-1 through the 60% ammonium sulfate precipitation step (Jones and Ballou, 1969). Bio-Gel P-2 (−400 mesh) was obtained from Bio-Rad Corp. and DEAE-Sephadex A-25 from Pharmacia.

General Procedures. Analytical procedures were employed for total carbohydrate (Stewart *et al.*, 1968), total and inorganic phosphate (Bartlett, 1959), reducing sugar (Paleg, 1959), and total nitrogen (Long and Staples, 1961). Vicinal glycols were detected by reacting a sample with a standard sodium metaperiodate solution and reading absorbance at 230 nm after 6 hr. Triose phosphates were detected by the method of Beck (1957). Glyceraldehyde and glyceraldehyde 2-phosphate were used as standards. Periodate oxidation of phosphomannan was followed by assaying periodate with 2,4,6-tri-2-pyridyl-2-triazine (Avigad, 1969).

Descending paper chromatography was carried out on Whatman No. 1 filter paper using the following solvent systems (in volume ratios): (A) 1-butanol–pyridine–water (3:1:1, v/v), (B) ethyl acetate–pyridine–water (8:2:1, v/v), (C) ethyl acetate–pyridine–water (10:4:3, v/v), (D) *tert*-butyl alcohol–water–

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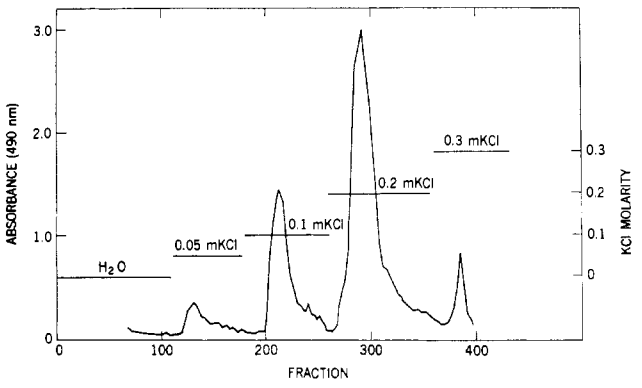

FIGURE 1: Elution pattern of the carbohydrate extract from 800 g of dried *K. brevis* cells on a DEAE-Sephadex A-25 column (4 × 200 cm). Stepwise elution was carried out with water, 0.05, 0.1, 0.2, and 0.03 M KCl. The four peaks are phosphodiester mannans.

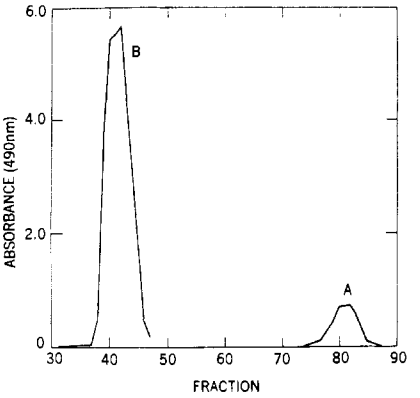

FIGURE 2: Gelfiltration on a Bio-Gel P-2 column (2 × 200 cm) of *K. brevis* phosphodiester mannan-8.6 after treatment with 0.1 N HCl at 100° for 30 min. Peak A is mannose and peak B is phosphomonoester mannan.

picric acid (80:20:4, v/v/w). Paper electrophoresis was carried out on Whatman No. 1 paper in 0.05 M sodium tetraborate at 1000 V for 2 hr.

Sugars and sugar alcohols were detected on paper chromatograms with the silver nitrate–sodium hydroxide or periodate–benzidine reagents (Gordon *et al.*, 1956), phosphate according to the method of Bandurski and Axelrod (1951), and amino acids by the ninhydrin spray of Wiggins and Williams (1952).

Proton magnetic resonance spectra were measured at room temperature with Varian A-60 and HR-220 spectrometers using tetramethylsilane as a standard. Samples were lyophilized three times from 99.5% D_2O, dried over P_2O_5 and dissolved in D_2O. The areas of nuclear magnetic resonance peaks were measured with a compensating polar planimeter.

^{31}P nuclear magnetic resonance spectra were measured with a Fourier transformed spectrometer at 31°. Heavy metals were removed from solution by Chelex-100 (Na^+) or EDTA yielding solutions with a pH of 9.3.

Optical rotation was measured with a Series 1100 Bendix Automatic polarimeter. Absorbances were read on a Zeiss PMQ spectrophotometer and a Nuclear-Chicago Unilux II scintillation counter was used to determine the radioactivity of labeled samples. pH measurements and titration curves were determined on a Radiometer pH meter 4. The amino acid content of *S. cerevisiae* mannans was measured with a Beckman Model 120C Automatic amino acid analyzer.

Acetylation and acetolysis of mannans was performed according to Kocourek and Ballou (1969). After termination of the acetolysis reaction with pyridine, the solution was evaporated to dryness under vacuum. Deacetylation was accomplished by suspending the residue in a sodium hydroxide solution maintained at pH 12.5 for 45 min. The solution was neutralized with solid CO_2. Methylation and gas chromatography procedures have been described (Stewart *et al.*, 1968).

Results and Discussion

Purification of K. brevis Phosphodiester Mannan. The crude carbohydrate-containing extract of *K. brevis* cells was exhaustively dialyzed, and then passed through a DEAE-Sephadex A-25 column (Cl^- form). Material retained on the column was divided into four fractions by stepwise elution with 0.05, 0.1, 0.2, and 0.3 M KCl. The elution pattern thus obtained for material isolated from log-phase cells (6-hr growth) is shown in Figure 1. The pattern was not changed if the phosphate content of the growth medium was increased, or if cells were harvested in stationary phase (70-hr growth). Material isolated from log-phase cells was used in all subsequent work. Each peak in Figure 1 was pooled and passed through a Dowex 50-X12 column (hydrogen form) by elution with water. The material, unretarded by the Dowex 50 column and giving a positive test for carbohydrate, was dialyzed and lyophilized. The four products are referred to hereafter by their mannose to phosphate ratio: phosphomannan-20 (eluted by 0.05 M KCl), phosphomannan-13.6 (eluted by 0.1 M KCl), phosphomannan-8.6 (eluted by 0.2 M KCl), and phosphomannan-6.5 (eluted by 0.3 M KCl). A total of 10 g of these mannans was obtained from 800 g of dried cells. After hydrolysis in 2 N HCl for 4 hr at 100°, paper chromatography in solvents A and C and paper electrophoresis revealed the presence of mannose, mannose 6-phosphate, traces of glucosamine, and several other ninhydrin-positive compounds. The nitrogen content of phosphomannan-8.6 was 0.96%.

Acid Cleavage of the Phosphodiester Bond in K. brevis Phosphodiester Mannan. Titration of the phosphomannans with 0.0025 N NaOH showed an uptake of base in the region pH 5.0–8.0 equal to only 0.1 equiv/phosphate. After treatment of the phosphomannan with 0.1 N HCl at 100° for 30 min, one equivalent of base per phosphate was consumed in this pH region. This indicates that the phosphate was diesterified in the isolated phosphomannans.

The mannan treated with 0.1 N HCl was chromatographed on a Bio-Gel P-2 column. In Figure 2, the excluded peak B is the high molecular weight phosphomannan with monoesterified phosphate, while peak A was shown by paper chromatography to be mannose. No inorganic phosphate was detected. Further treatment of peak B with 0.1 N HCl under the above conditions released negligible mannose. The ratio of peak B to A was 7.3 for phosphodiester mannan-8.6, while the mannose to phosphate ratio for the peak B material was 7.2. Thus, one equivalent of mannose was released from the mannan when the phosphodiester was cleaved. Peak B material is referred to hereafter as phosphomonoester mannan. A similar treatment of phosphodiester mannan-6.5, gave a phosphomonoester mannan with a M:P ratio of 5.6.

Determination of the Linkage of Mannose to Phosphate

Kinetics of Phosphodiester Cleavage. The release of mannose from the phosphodiester mannan was measured by the increase in reducing sugar. Buffers were prepared with pH values

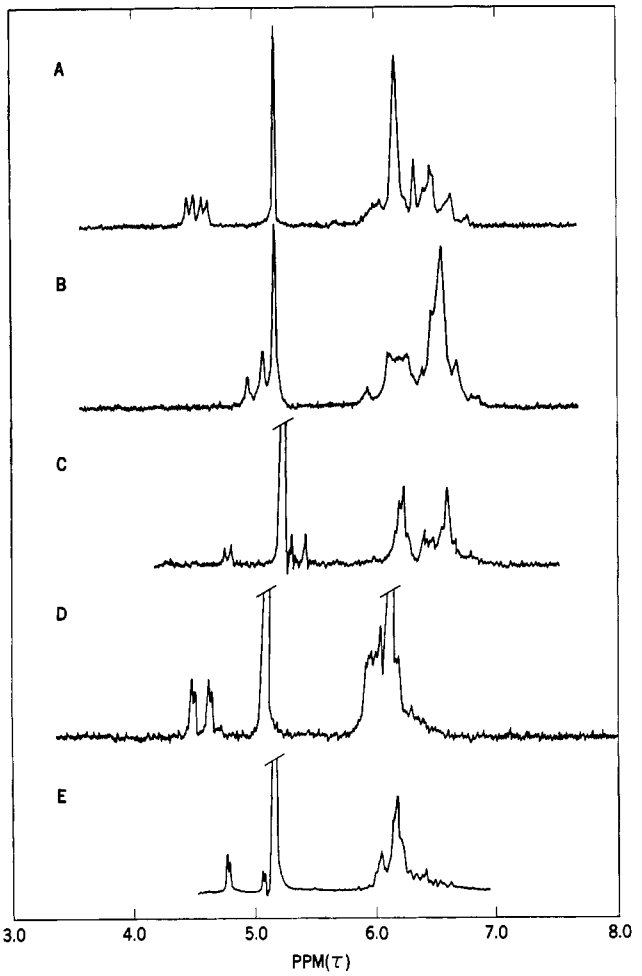

FIGURE 3: Proton magnetic resonance spectra of α-D-glucose 1-phosphate (A), β-D-glucose 1-phosphate (B), the anomeric mixture of D-glucose (C), α-D-mannose 1-phosphate (D), and the anomeric mixture of D-mannose (E). Spectra were obtained with a Varian A-60 spectrometer at pH 8.6 with tetramethylsilane as an external standard.

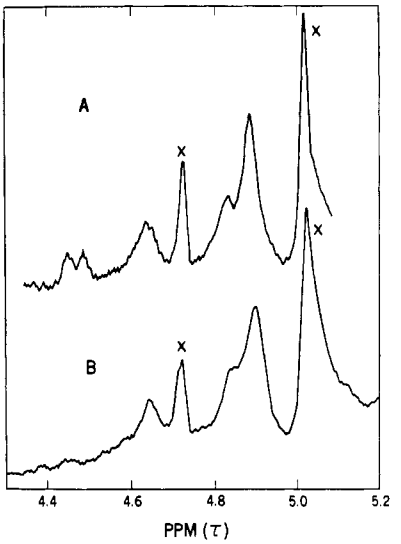

FIGURE 4: The 220-MHz nuclear magnetic resonance spectra of the anomeric protons of *K. brevis* phosphodiester mannan-6.5 (A) and its corresponding phosphomonoester mannan (B) at pH 8.6. The peak τ 4.5, assigned to the anomeric proton of mannose linked to phosphate through position 1, is present in the diester but absent in the monoester. Peaks at τ 4.65, 4.85, and 4.9 were assigned to the other anomeric protons of the mannan. Peaks labeled "×" were shown to be spinning side bands of the HDO signal.

at 100° of 2.1, 2.45, and 3.15 according to Stene (1930). Half-lives for the release of mannose were 3.36 (pH 2.1), 7.2 (pH 2.45), and 35 min (pH 3.15). This rate of hydrolysis is considerably faster than one would expect for the hydrolysis of a phosphodiester in the presence of a *vic*-glycol (Cox and Ramsey, 1964) and, in keeping with the known instability of glycosyl phosphates (Leloir and Cardini, 1957), indicates that mannose was attached to phosphate through position 1.

Proton Magnetic Resonance of Phosphodiester Mannan. Further evidence that mannose was attached to phosphate through position 1 was obtained from the high-resolution proton magnetic resonance spectra of the anomeric protons of the phosphodiester mannan. To demonstrate the effect of phosphorylation at position 1 on the signal of the anomeric proton of hexoses, α-D-glucose 1-phosphate, β-D-glucose 1-phosphate, and α-D-mannose 1-phosphate were used as model compounds. As shown in Figure 3, the chemical shifts of the anomeric protons of α-D-glucose 1-phosphate and β-D-glucose 1-phosphate were 0.2 and 0.3 ppm downfield from the respective anomeric protons of the α and β anomers of free glucose. The axial C-1 proton of β-D-glucose 1-phosphate gave a triplet at τ 5.1 with J = 7 Hz, a result of coupling both to the axial C-2 hydrogen and the phosphorus. The α-D-glucose 1-phosphate equatorial C-1 proton gave a quartet at τ 4.55, coupled to the C-2 hydrogen (J = 3 Hz) and to phosphorus (J = 8 Hz). The C-1 proton of α-D-mannose 1-phosphate also gave a quartet at τ 4.55 (0.2 ppm downfield from the α-D-mannose C-1 proton) which was coupled to the C-2 hydrogen (J = 1.5 Hz) and to phosphorus (J = 8 Hz). The magnitudes of the deshielding and splitting by phosphorus were similar to values that have been reported for alkyl phosphate esters (Mavel, 1966).

The proton magnetic resonance spectra of the anomeric proton region of phosphodiester mannan-6.5 and the corresponding phosphomonoester mannans are shown in Figure 4. The phosphodiester mannan gave a doublet at τ 4.5 (J = 8 Hz) which was absent in the phosphomonoester mannan. In light of our observations on the spectra of glycosyl 1-phosphates, we assign this signal to the anomeric proton of the mannose linked to phosphate. The peaks at τ 4.65, 4.85, and 4.9 are assigned to the other anomeric protons on the mannan. The ratio of the peak at τ 4.5 to the other anomeric peaks was 1:7.6. This is close to the value of 1:5.5 that is predicted for a phosphodiester mannan in which one out of every 6.5 mannose units is linked to phosphate through position 1.

Since the nonphosphate-linked anomeric signals occurred at positions one would expect for α-linked mannose units (Lee and Ballou, 1965), and since the observed specific rotation $[\alpha]_{546}$ for this mannan was +75°, we conclude that the phosphodiester mannan contains mainly α linkages. Since the phosphate-linked mannose in the mannan gave an anomeric signal 0.2–0.3 ppm downfield from the other anomeric signals, we conclude that this mannose too has the α configuration. Because the τ 4.5 peak only accounted for 75% of the phosphate-linked mannose, we cannot say that all of the linkages to phosphate are α.

Identification of the Second Group Esterified to Phosphate by Smith Degradation of the Mannan. Although mannose 6-phosphate is released on total hydrolysis of the phosphodiester mannan, we considered the possibility that phosphate

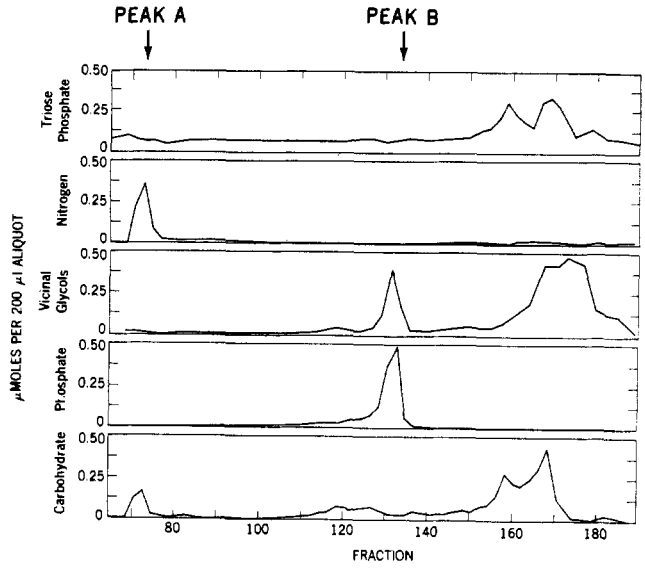

FIGURE 5: Gel filtration, on a Bio-Gel P-2 column (2 × 200 cm) eluted with 0.1 M KCl, of the products of Smith degradation of *K. brevis* phosphodiester mannan-8.6. Assays were performed for carbohydrate, phosphate, *vic*-glycols, nitrogen, and triose phosphate. The material in fractions 150–180, which was a mixture of alcohols and aldehydes, gave a positive reaction in the triose phosphate assay although it contained no phosphate.

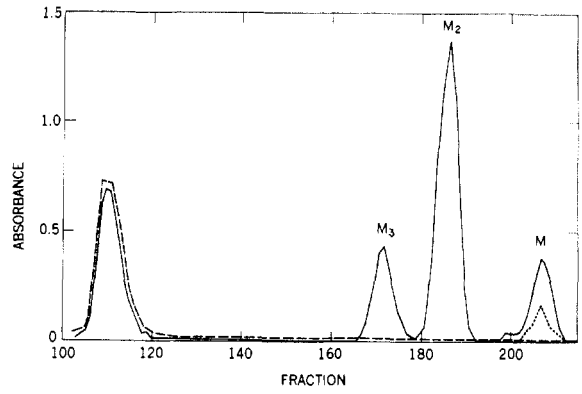

FIGURE 6: Gel filtration on a Bio-Gel P-2 column (2 × 200 cm) of the products of acetolysis of *K. brevis* phosphodiester mannan-8.6 and its corresponding phosphomonoester mannan. The pattern obtained for the phosphomonoester mannan was nearly identical with that of the phosphodiester mannan except for peak M. Peak M for the phosphomonoester mannan is shown as a dotted line. Assays were performed for carbohydrate (solid and dotted lines) and for phosphate (dashed line). The phosphate-negative peaks are mannotriose (M_3), mannobiose (M_2), and mannose (M). Only one phosphate-containing peak was obtained. The molar ratios of the latter, shown to be a phosphorylated trisaccharide, to M_3:M_2:M are 2.08:1:5:3 for the diester, and 2.15:1:5.2:1.3 for the monoester.

could migrate during hydrolysis and actually be present at some other position in the intact mannan. For this reason a Smith degradation (Goldstein *et al.*, 1965), of the mannan was performed. In this reaction, mannose 6-phosphate which was glycosidically linked should yield glycerol 1-phosphate, while mannose 4-phosphate, mannose 3-phosphate, and mannose 2-phosphate would give erythritol 2-phosphate, mannose 3-phosphate, and glyceraldehyde 2-phosphate, respectively.

Phosphodiester mannan-8.6 (200 mg) consumed 1.3 equiv of periodate/mannose unit after 80 hr at pH 4.0. Only a slight further reaction was observed after 50 additional hr. Excess periodate was destroyed with ethylene glycol and the oxidized phosphodiester mannan was desalted on a Sephadex G-25 column. All phosphate-containing material was eluted in the void volume of the column. This material was reduced with 1 g of sodium borohydride for 10 hr, and the product was desalted on Sephadex G-25. Again all phosphate was in the excluded peak. The last trace of borate was removed by evaporation under vacuum from 0.3% methanolic HCl, and hydrolysis was carried out in 0.5 M HCl for 12 hr at room temperature. The hydrolysate was neutralized and applied to a Bio-Gel P-2 column, and then eluted with 0.1 M KCl. The elution pattern is shown in Figure 5. An excluded peak was obtained (peak A) which contained carbohydrate and nitrogen but no phosphate. This, presumably, was the peptide moiety that is known to be attached to yeast cell wall mannans (Sentandreu and Northcote, 1968). Only one phosphate-containing peak was found (peak B). As shown in Figure 5, the material in the phosphate-containing peak consumed periodate but did not give a positive reaction in the triose phosphate assay, or phenol–sulfuric assay for carbohydrate. Therefore, the phosphate-containing compound was neither mannose phosphate nor glyceraldehyde 2-phosphate. Paper chromatography of the compound in solvent C showed only glycerol phosphate to be present. No erythritol phosphate was found. Dephosphorylation of the fragment with alkaline phosphatase gave glycerol, identified by paper chromatography in solvent A. No erythritol or glyceraldehyde was found. We conclude that the second group to which phosphate was esterified in the phosphodiester mannan was position 6 of a mannose residue in the polysaccharide.

Position of the Mannose 6-Phosphate Group in K. brevis Phosphodiester Mannan

Preparation of Phosphorylated Oligosaccharides. As shown by Stewart and Ballou (1968), the preferential cleavage of 1→6 linkages during acetolysis of *K. brevis* phosphomannan yields a mixture of phosphorylated and neutral oligosaccharides. The gel filtration patterns of the products of acetolysis of phosphodiester mannan-8.6 and the corresponding phosphomonoester mannan, are shown in Figure 6. As expected if the acetolysis procedure cleaved the phosphodiester bond to release mannose, the acetolysis pattern of the phosphomonoester mannan gave 60% less mannose than the pattern of the phosphodiester mannan. Figure 7 shows the acetolysis patterns obtained for phosphodiester mannan-6.5 and phosphodiester mannan-20. There was no change in the proportions of the neutral oligosaccharides with increasing phosphate content other than the expected increase in free mannose derived from the cleavage of the phosphodiester during acetolysis.

Acetolysis was carried out on phosphodiester mannan-8.6 for 6, 13, and 36 hr, and the phosphorylated oligosaccharides from each acetolysis were applied to DEAE-Sephadex A-25 column (HCO_3 form). The results are shown in Figure 8. Upon dephosphorylation with alkaline phosphatase, peaks M_3P and M_2P of Figure 8 yielded mannotriose and mannobiose, respectively, as demonstrated by paper chromatography in solvent A. Methylation analysis of the neutral oligosaccharide obtained from M_3P confirmed the 1→2 linkage already established by Stewart and Ballou (1968). Since the ratio M_2P:M_3P increased with the time of acetolysis, and since M_2P was absent in the 6-hr acetolysate, we conclude that M_2P was a degradative product of M_3P. The possibility that the neutral mannotriose, obtained during acetolysis, was a degradation

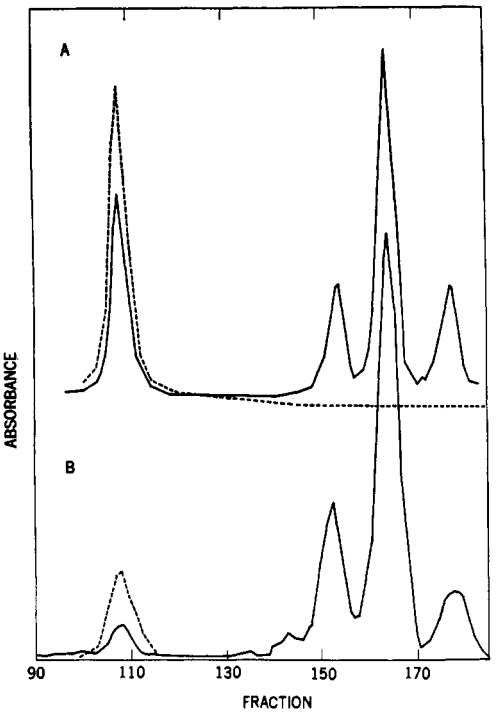

FIGURE 7: Gel filtration on a Bio-Gel P-2 column (2 × 200 cm) of the products of acetolysis of *K. brevis* phosphodiester mannan-6.5 (A) and phosphodiester mannan-20 (B). Assays were performed for carbohydrate (solid line) and phosphate (dashed line).

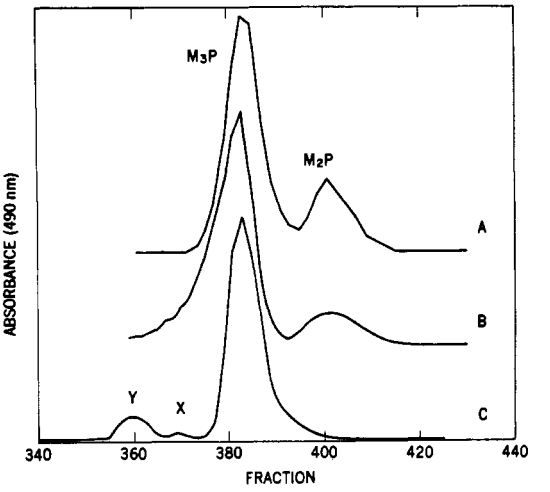

FIGURE 8: Chromatography of the phosphorylated oligosaccharides obtained by acetolysis of *K. brevis* phosphodiester mannan-8.6 on a DEAE-Sephadex A-25 column (2 × 200 cm) (HCO_3^- form). A linear gradient of 0–0.5 M NH_4HCO_3 was used (1 l. in each chamber) followed by elution with 0.5 M NH_4HCO_3. The phosphorylated oligosaccharides were eluted during the 0.5 M NH_4HCO_3 wash. Curves A, B, and C represent the products obtained by acetolysis for 36, 13, and 6 hr, respectively, The peaks labeled X and Y in the 6-hr acetolysis were not identified and may represent phosphorylated fragments with intact 1→6 linkages. No M_2P peak is evident in the 6-hr acetolysis. The molar ratios of M_3P to M_2P for the 13- and 36-hr acetolysates are 4.7 and 3.04.

product of M_3P is ruled out because inorganic phosphate was not produced in the acetolysis reactions.

Position of the Phosphate in M_3P. M_3P (100 mg), which had been reduced with an equal weight of sodium borohydride, was used in this experiment. A Smith degradation was carried out under the same conditions as were used for the whole mannan except that salts were removed after the acid hydrolysis step by gel filtration on Bio-Gel P-2. This yielded only one phosphate-containing peak which was phenol–sulfuric acid negative and periodate positive. After dephosphorylation with alkaline phosphatase, this material yielded only glycerol, as demonstrated by paper chromatography in solvent A. Thus, as in the whole mannan, the phosphate was esterified to position 6 of a mannose in M_3P.

Further proof that the phosphate was attached to a primary hydroxyl was obtained from the ^{31}P nuclear magnetic resonance spectrum of M_3P. Figure 9 shows the spectrum compared to those of standard D-mannose 6-phosphate and D-glucose 1-phosphate. Owing to phosphorus–proton coupling, D-mannose 6-phosphate gave a triplet while D-glucose 1-phosphate gave a doublet. Although complete chemical homogeneity was not demonstrated by this technique, the partially resolved triplet given by M_3P indicates that phosphate was esterified to a primary hydroxyl.

Position of the Mannose 6-Phosphate Residue in the Phosphorylated Oligosaccharides. To determine the position of mannose 6-phosphate in M_3P, 50 mg of M_3P (contaminated with a small amount of M_2P produced during acetolysis) was reduced with sodium borotritide and hydrolyzed in 1 N HCl for 3 hr at 100°. Paper chromatography of the products in solvent B and paper electrophoresis revealed the presence of mannose, mannitol, and a phosphorylated sugar in approximately equal amounts. Gel filtration of the product on a Bio-Gel P-2 column gave the pattern shown in Figure 10. Peaks B and C were assigned to mannitol and mannose, respectively (mannitol gives no color in the phenol–sulfuric acid assay). This column was shown to separate a standard mixture of mannose and mannitol, with mannitol eluting in the position of a disaccharide. Since the bulk of the radioactivity was in the phosphate-negative, mannitol-containing peak B, it was concluded that mannose 6-phosphate was not at the reducing end of M_3P, otherwise [^{3}H]mannitol phosphate would be formed. The small amount of radioactivity in the phosphate-containing peak A was probably due to the presence of some M_2P which, as will be shown later, has some of its phosphorylated sugar at the reducing end. The fact that the sugar phosphate peak showed the same reducing sugar equivalent as the mannose peak also indicated that mannose 6-phosphate was not attached to the reducing end of M_3P. The several minor

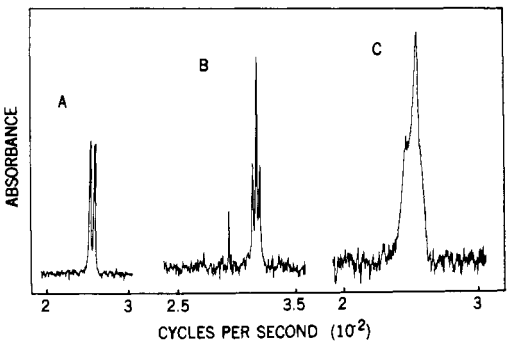

FIGURE 9: ^{31}P (24.287 MHz) nuclear magnetic resonance of α-D-glucose 1-phosphate (A), D-mannose 6-phosphate (B), and mannotriose phosphate (M_3P) derived from *K. brevis* phosphodiester mannan by acetolysis (C). Standard 85% phosphoric acid gave a signal at −200 cycles/sec.

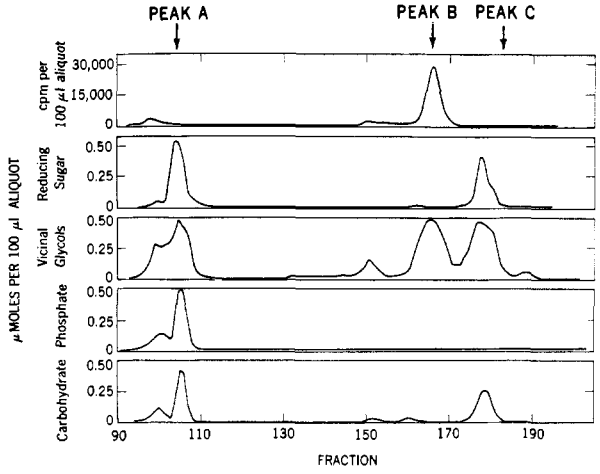

FIGURE 10: Gel filtration on a Bio-Gel P-2 column (2 × 200 cm) of the products of total acid hydrolysis of sodium borotritide reduced M_3P. Assays were performed for carbohydrate, phosphate, *vic*-glycols, reducing sugar, and radioactivity.

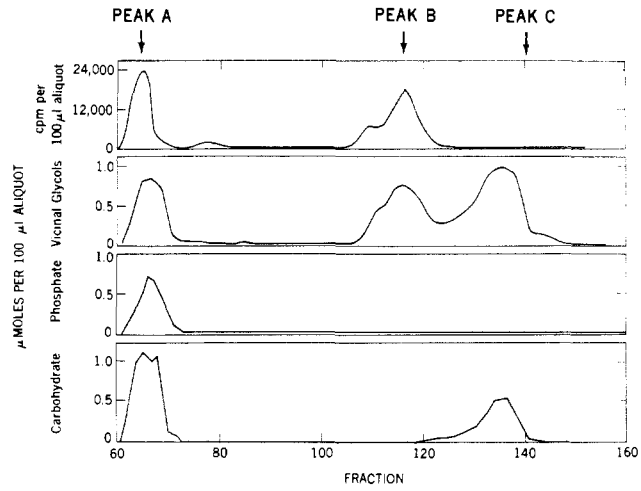

FIGURE 12: Gel filtration of the products of partial acid hydrolysis of sodium borotritide reduced M_3P. Assays were performed for carbohydrate, phosphate, *vic*-glycols, and radioactivity.

peaks in Figure 10 were not identified, but they may represent partial hydrolysis products.

To determine whether the phosphate was located on the middle sugar or at the nonreducing end of M_3P, the substance was reduced with sodium borotritide and the product was subjected to partial acid hydrolysis in 0.33 N HCl at 100°. As shown in the scheme in Figure 11, different products should result from the two alternative structures for reduced M_3P. The hydrolysis was stopped after 5.5 hr when 50% of the glycosidic bonds had been cleaved as determined by a reducing sugar assay. The product was passed over a Bio-Gel P-2 column to give the pattern shown in Figure 12. Peaks B and C were assigned to mannitol and mannose, respectively. Since a neutral peak was not found which contained both carbohydrate and radioactivity, we conclude that fragment II in Figure 11 was not produced. This indicates that the mannose 6-phosphate unit was not at the nonreducing terminus in M_3P.

The phosphate-containing peak A of Figure 12 was chromatographed on a DEAE-Sephadex A-25 column (HCO_3^- form) to give the pattern shown in Figure 13. Peaks A, B, and C cochromatographed on paper in solvent D with mannose 6-phosphate, M_2P, and M_3P, respectively. The fact that peak B had radioactivity, carbohydrate and phosphate, indicates that it contained some fragment IV of Figure 11. Since such a fragment could not be formed if the mannose 6-phosphate were at the nonreducing terminus, we conclude that the phosphate was on the middle sugar of M_3P. Further evidence for the composition of peak B in Figure 13 was obtained by total hydrolysis of this material followed by passage through a DEAE-Sephadex column which gave the pattern shown in the top of Figure 14. As would be expected for the hydrolysis of a mixture of

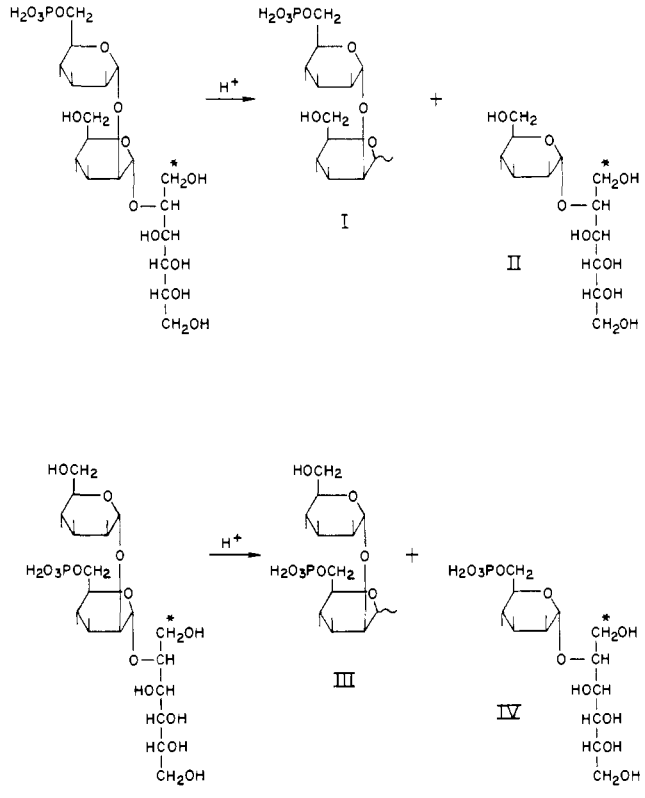

FIGURE 11: The expected disaccharide products resulting from the partial acid hydrolysis of the two possible structures for reduced M_3P. In both cases, mannose, mannose 6-phosphate, and mannitol, as well as starting material, should be produced.

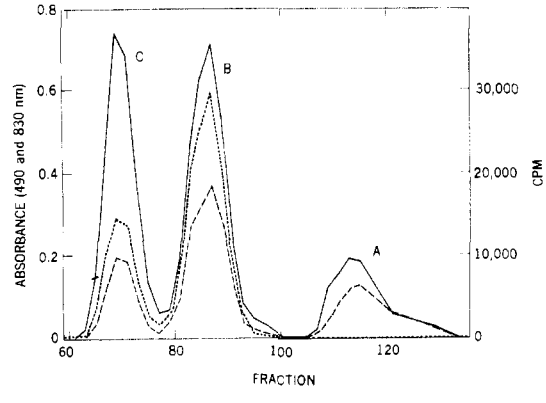

FIGURE 13: Chromatography on a DEAE-Sephadex A-25 column (2 × 200 cm) of the phosphate-containing peak from Figure 12. Elution with NH_4HCO_3 is the same as for Figure 8. Assays were performed for carbohydrate (solid line), phosphate (dashed line), and radioactivity (dotted line).

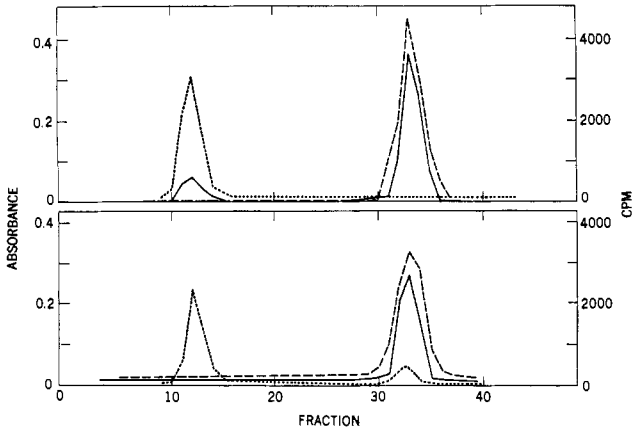

FIGURE 14: Chromatography on DEAE-Sephadex A-25 of the total hydrolysis products (1 N HCl at 100° for 4 hr) of the peak B material of Figure 13 (top), and the total hydrolysis products of sodium borotritide reduced peak B material (bottom). Elution was carried out in a stepwise manner with water and 0.5 M NH_4HCO_3. Assays were performed for carbohydrate (solid line), phosphate (dashed line), and radioactivity (dotted line).

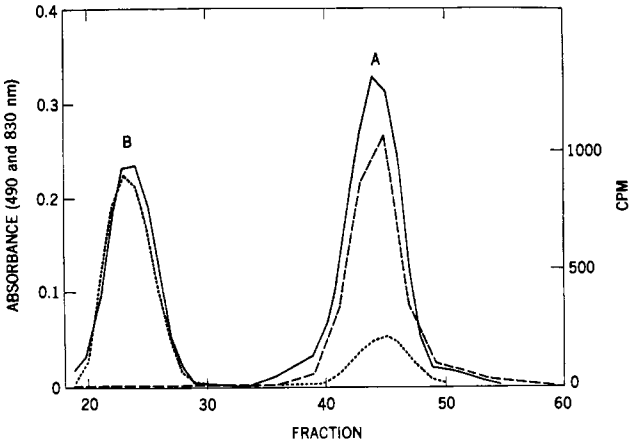

FIGURE 15: Chromatography on a DEAE-Sephadex A-25 column (2 × 40 cm) of the products of total acid hydrolysis of sodium borotritide reduced M_2P. Elution was carried out in a stepwise manner with water and 0.5 M NH_4HCO_3. Assays were performed for carbohydrate (solid line), phosphate (dashed line), and radioactivity (dotted line). NH_4HCO_3 did not interfere with the phenol–sulfuric acid assay for carbohydrate or appreciably quench the radioactivity.

fragments III and IV (Figure 11), a radioactive, phosphate-negative, carbohydrate-containing peak was detected (mannose and mannitol) as well as a phosphate-containing, nonradioactive carbohydrate (mannose phosphate, but no mannitol phosphate). After reduction of the peak B material of Figure 13 with sodium borotritide, followed by total hydrolysis and chromatography on DEAE-Sephadex, the pattern at the bottom of Figure 14 was obtained. Here, no carbohydrate was found in the radioactive neutral fraction, indicating only the presence of mannitol. The phosphate-positive fraction now contained radioactivity, indicating mannitol phosphate. This was the result expected for a mixture of fragments III and IV after reduction.

M_2P has been shown to be a degradative product of M_3P formed during acetolysis. To determine whether the phosphate was esterified to the reducing or nonreducing sugar of this disaccharide, 20 mg of M_2P was reduced with sodium borotritide, and the product was hydrolyzed in 1 N HCl for 4 hr at 100°. The mixture was passed through a DEAE-Sephadex A-25 column and eluted stepwise with water and 0.5 M NH_4HCO_3, to give the pattern shown in Figure 15. Since peak B contained both radioactivity and phenol–sulfuric acid-positive carbohydrate, it was a mixture of mannose and mannitol. Peak A contained phosphate, phenol–sulfuric acid-positive carbohydrate, and radioactivity, indicating it was a mixture of mannose phosphate and mannitol phosphate.

Since both mannitol and mannitol phosphate were obtained in this experiment, M_2P must be a mixture of two isomers, one with the phosphate on the reducing mannose unit, the other having phosphate on the mannose at the nonreducing end. The ratio of the two isomers was calculated from the ratios of carbohydrate and radioactivity in peak A to those in peak B of Figure 15. Correction was made for an observed 21% conversion of mannose 6-phosphate into mannose under the conditions of the experiment, and the same rate of hydrolysis was assumed for mannitol phosphate. The results indicate that M_2P contained 25–27% of the isomer with the phosphate on the mannose unit at the reducing end. Apparently, a preferential cleavage of M_3P occurred during acetolysis because of the proximity of the phosphate group to the glycosidic linkage at position 2 of the same mannose unit.

Action of α-Mannanase GJM-1 on K. brevis Phosphodiester Mannan. Phosphodiester mannan-8.6 was incubated with α-mannanase according to Jones and Ballou (1969). After digestion for 24 hr, reducing sugar (mannose) reached a constant value of 50% of the original carbohydrate. The high molecular weight fraction, which was resistant to the further action of the enzyme, was separated from the mannose by gel filtration on Sephadex G-25 and from the enzyme by treatment with Dowex 50 (H^+). Examination of the proton magnetic resonance spectrum of the product showed a signal at τ 4.5, which is characteristic of an intact mannose 1-phosphate linkage. Mild acid hydrolysis (0.1 M HCl, 100° for 30 min) released 0.8 equiv of mannose/phosphate. Thus, the α-mannanase did not cleave the phosphodiester linkage to release mannose.

Acetolysis was performed on the α-mannanase-resistant fraction, and the phosphorylated oligosaccharides were isolated as described before. Dephosphorylation with alkaline phosphatase produced only mannobiose, identified by paper chromatography in solvent C, indicating that the α-mannanase removed the terminal mannose from the phosphorylated side chains in *K. brevis* phosphodiester mannan.

Investigation of the Phosphate Ester in S. cerevisiae Mannan Isolation. Mannan was isolated from *S. cerevisiae* cells, harvested in log phase, in the manner described for *K. brevis*. Dialyzed crude carbohydrate material was put onto a DEAE-Sephadex A-25 (Cl^-) column and eluted in a stepwise manner with water and 0.2 M KCl. Fom the peak eluted with water, about 2.4 g of mannan (mannose to phosphate ratio of 290) was precipitated by Fehling's solution (Stewart *et al.*, 1968). After Dowex 50 (H^+) treatment, 0.4 g of mannan, mannose to phosphate ratio of 20, was obtained from the material eluted by 0.2 M KCl.

Acid Treatment of S. cerevisiae Phosphomannan. Mild acid hydrolysis of *S. cerevisiae* phosphomannan was carried out either by heating in 0.01 N HCl solution at 100° for 1 hr, or by heating the free acid form of the phosphomannan, obtained by treatment with Dowex 50 (H^+), at 100° for 20 min. Both products, when chromatographed on a Bio-Gel P-2 column, gave the pattern shown in Figure 16A. The low molecular weight peaks were identified as mannose and mannobiose, present in

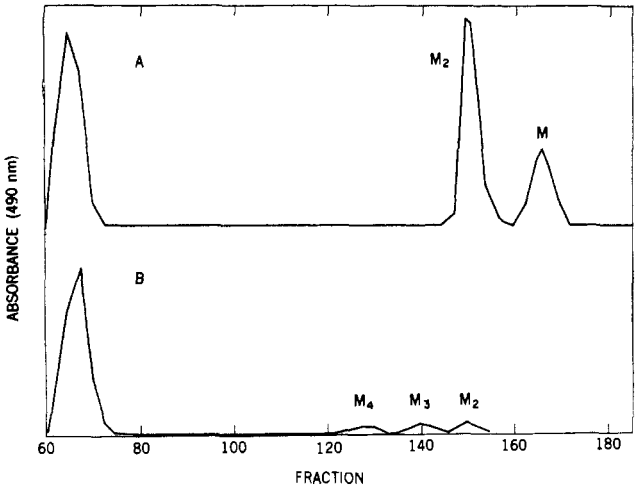

FIGURE 16: Gel filtration on a Bio-Gel P-2 column (2 × 200 cm) of the oligosaccharides released from *S. cerevisiae* phosphomannan by mild acid hydrolysis (A), and from this acid-treated phosphomannan by alkaline β-elimination conditons (B). Aliquots used in the phenol–sulfuric acid assay were 50 μl for fractions 60–74 and 1 ml for fractions 75–180. The included peaks are mannose and mannobiose (A), and mannobiose, mannotriose, and mannotetraose (B).

approximately equimolar amounts. The molar ratio of mannose in these sugars to that in the high molecular weight product was 1 to 12.5, the value expected if equal amounts of mannose and mannobiose were glycosidically linked to the phosphate in a phosphomannan with a mannose to phosphate ratio of 20.

Oligosaccharides Obtained by Alkaline Treatment of S. cerevisiae Phosphomannan. It has been shown that sugars linked to serine and threonine in glycoproteins are more sensitive to acid hydrolysis than sugars linked by other glycosidic linkages (Kochetkov *et al.*, 1967). Since oligosaccharides linked to serine and threonine occur in *S. cerevisiae* mannan (Sentandreu and Northcote, 1968), it was important to show that the mannose and mannobiose released upon mild acid hydrolysis of *S. cerevisiae* mannan did not originate from sugars linked to these amino acids. Therefore, *S. cerevisiae* phosphomannan,

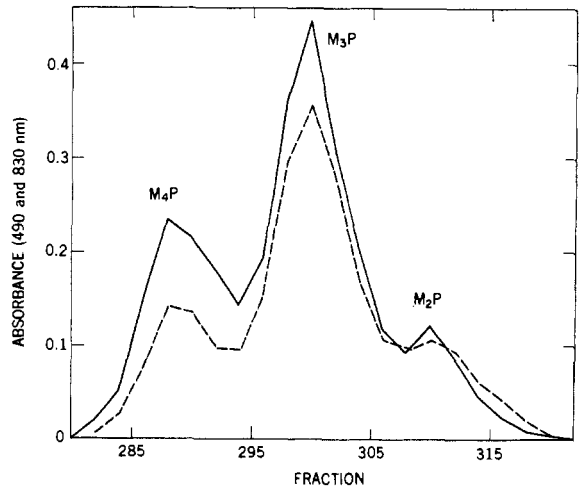

FIGURE 17: Chromatography on a DEAE-Sephadex A-25 column (2 × 200 cm) of the phosphorylated products of acetolysis of *S. cerevisiae* phosphomannan. Conditions were the same as for Figure 8. Assays were performed for carbohydrate (solid line) and phosphate (dashed line).

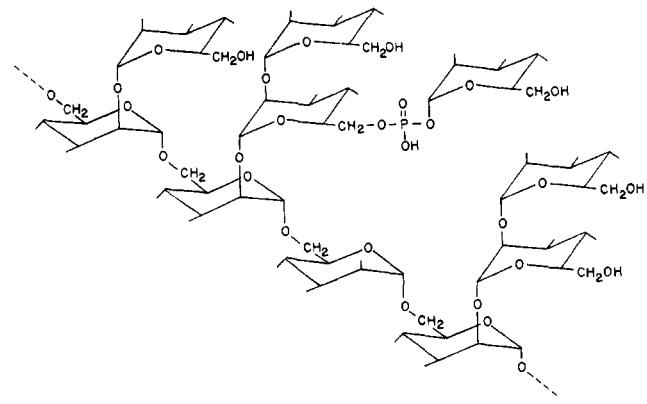

FIGURE 18: Proposed structure for the phosphodiester linkage in *K. brevis* mannan. The sequence of the side chains is unknown and may be random.

which had been heated twice with acid to remove mannose and mannobiose, was subjected to alkaline β-elimination conditions. The phosphomonoester mannan was treated with 0.25 N NaOH at 4° for 18 hr under N_2, and the product was neutralized with Dowex 50 (H^+) and chromatographed on a Bio-Gel P-2 column (Figure 16B). The oligosaccharides released were identified (by paper chromatography in solvent C) as mannobiose, mannotriose, and mannotetraose. No mannose was detected. Examination of the high molecular weight phosphomannan after alkaline treatment showed a loss of threonine (0.33 mole/250 mannose units) and of serine (0.44 mole/250 mannose units), which more than accounted for the oligosaccharides released from the mannan by β elimination (0.5 mole/250 mannose units).

Since the sugars released by acid treatment of *S. cerevisiae* phosphomannan had a composition different from those released by β elimination, we conclude that they were not attached to serine and threonine but instead were linked to phosphate. This conclusion is supported by the fact that the amount released corresponded to the phosphate content of the mannan.

Determination of the Position of Phosphate Esterification in the Mannan. Acetolysis was performed on *S. cerevisiae* phosphomannan by the procedure used for *K. brevis*. Gel filtration of the products of acetolysis gave a phosphate-containing peak plus a pattern of neutral oligosaccharides identical to that found by Kocourek and Ballou (1969). The phosphate-containing material was chromatographed on a DEAE-Sephadex A-25 column to give the pattern in Figure 17. Dephosphorylation of the peaks in Figure 17 with alkaline phosphatase yielded mannobiose, mannotriose, and mannotetraose as demonstrated by paper chromatography in solvent C. Since the amount of M_2P was small it was probably a degradation product of M_3P formed during acetolysis, as was shown for *K. brevis* phosphomannan. Thus, phosphate is esterified to the tri- and tetrasaccharide units in *S. cerevisiae* phosphomannan, but probably not to the disaccharide unit.

Conclusions

Stewart and Ballou (1968) demonstrated that *K. brevis* phosphodiester mannan consisted of a backbone of α-1→6-linked D-mannose residues substituted by α-1→2-linked side chains of one and two D-mannose residues. The experiments described in this report demonstrate that single α-D-mannose residues are esterified through position 1 to the phosphate group in *K. brevis* phosphodiester mannan. The phosphate, in turn, is esterified

to position 6 of the middle D-mannose residue in trisaccharide units obtained through selective cleavage by acetolysis of the 1→6 linkages in the backbone. Phosphate does not occur in the analogous disaccharide units in the mannan. From these results we can propose the structure in Figure 18 for the region of the polysaccharide near the phosphodiester units. The preliminary conclusion (Stewart and Ballou, 1968) that phosphate was esterified to position 3 or 4 of a mannose in the backbone of the mannan was probably incorrect.

In a parallel study, it was found that equimolar amounts of mannose and mannobiose are esterified through position 1 to phosphate in *S. cerevisiae* phosphodiester mannan. The phosphate group, in turn, is esterified to tri- and tetrasaccharide units in the mannan. From these data, it appears that *S. cerevisiae* phosphomannan is analogous to that of *K. brevis*, although perhaps with some additional complexities.

K. brevis and *S. cerevisiae* cell wall phosphomannans have features in common with the exocellular phosphomannans from the yeasts *Hansenula capsulata* and *Hansenula holstii*, which consist of short oligosaccharides polymerized by α-1→6-phosphodiester linkages (Gorin and Spencer, 1968). A phosphorylated trisaccharide was isolated from *H. capsulata* exocellular mannan with a structure similar to that which we have determined for M_3P from *K. brevis* (Slodki, 1963). This raises the possibility that common enzymes are involved in the biosynthesis of these two different types of mannans. Yeast cell wall mannans are thought to be synthesized from GDP-mannose by means of a phosphorylundecaprenol-carrier lipid (Behrens and Cabib, 1968; Tanner, 1969; Kozak and Bretthauer, 1970). Stewart and Ballou (1968) have suggested that 1→2- and 1→3-linked oligosaccharides, first synthesized on this carrier lipid, may be polymerized to form the 1→6-linked backbone of yeast cell wall mannan. It is also possible that some oligosaccharides are transferred, along with the phosphate group from the carrier lipid to a specific residue in the mannan to produce the phosphodiester structures we have described. Alternatively, a phosphodiester linkage first could be formed between two oligosaccharides, followed by polymerization of one of them into the 1→6-linked backbone. If backbone formation were omitted, continued phosphodiester formation would produce the exocellular type of phosphomannan.

In addition to introducing surface charges with a possible role in yeast flocculation (Mill, 1966), the phosphodiesters of yeast cell wall mannan may provide cell surface specificity utilized in cell interaction during yeast mating. In the following paper (Raschke and Ballou, 1971) it will be shown that the phosphodiester structure is located on the cell surface in *K. brevis* and that it is the immunodominant group on the whole yeast cell.

Acknowledgments

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