Structure of the Mannan from *Saccharomyces* Strain FH4C, a Mutant Constitutive for Invertase Biosynthesis. I. Significance of Phosphate to the Structure and Refractoriness of the Molecule†

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ABSTRACT: The cell wall mannan from *Saccharomyces* strain FH4C (a yeast mutant which forms large amounts of the mannan–enzyme, invertase) was examined. The mannan can be fractionated into neutral and acidic components on DEAE-cellulose. Covalently bound phosphate is responsible for the acidic properties of the polysaccharide. Gel filtration studies indicate that the mannan has a molecular weight range from 40,000 to >300,000 daltons. The susceptibility of FH4C mannans to a bacterial α-mannosidase was inversely related to the content of mannan phosphate and was augmented by dephosphorylation of the polysaccharide. Thus, phosphate shields the mannan from enzymolysis by masking potentially susceptible mannosidic bonds. Mannans severely degraded by α-mannosidase were capable of reacting with concanavalin A, indicating that oligomannosidic branches persist in these mannan residues (*i.e.*, cores) after enzymolysis. Thus, the cores have a structure more complex than that of a linear α-1,6-linked polymannose chain. Mannan phosphate exists in the form of phosphodiester bonds. These are cleaved by dilute acid releasing mannose and mannobiose and simultaneously generating a mannan which is enriched in secondary phosphate and susceptible to alkaline phosphatases. The acid-labile ester bond is probably between phosphate and C-1 of mannose and/or mannobiose; the phosphorus which survives acid hydrolysis exists as a 6-phosphomannose residue, probably at the nonreducing end of a mannan chain.

The polysaccharide mannan accounts for 30–45% of the yeast cell wall and is comprised of D-mannose residues joined by α-1,2, α-1,3, and α-1,6 linkages. In baker's yeast mannan, these bonds are in the proportions 50:15:35 (Haworth *et al.*, 1937, 1941; Cifonelli and Smith, 1955; Peat *et al.*, 1961a,b). The polysaccharide has been depicted as having an arboreal structure with α-1,2- and α-1,3-linked mannosidic branches radiating from a "backbone" of mannose residues joined by α-1,6 bonds.

Most yeast mannans are quite large, ranging from 20,000 to 98,000 (Jones and Ballou, 1969b; Korn and Northcote, 1960). However, very high molecular weight mannans (200,000–500,000) have been prepared (McLellan and Lampen, 1968; Eddy and Longton, 1969; Thieme and Ballou, 1972).

Additional components of mannan are phosphorus and protein. The latter, which may account for 6% of the polymer (Thieme and Ballou, 1971) cross-links large mannan chains to form macromolecular complexes with molecular weights of up to 300,000 or more; the linkage between mannan and mannan–protein is probably an *N*-glycosidic bond between *N*-acetylglucosamine and asparagine (Sentandreu and Northcote, 1968). Mannan phosphate exists in the form of phosphodiester bonds which bridge residues of mannose and/or mannobiose to high molecular weight mannan chains (Thieme and Ballou, 1971).

In this paper, we have characterized the mannan from *Saccharomyces* strain FH4C, a yeast mutant constitutive for synthesis of invertase, an enzyme which consists of approximately equal amounts of mannan and protein (Neumann and Lampen, 1967; Gascón *et al.*, 1968). The susceptibility of FH4C mannan to a bacterial α-mannosidase is described. The mannosidase-resistant residues (*i.e.*, cores) were characterized and the component responsible for the refractoriness of mannan has been identified. Attempts to fully depolymerize the mannan *via* auxiliary agents are also described. The nature of the phosphate component of mannan has been partially elucidated.

Materials and Methods

Arthrobacter GJM-1, the source of α-mannosidase, was obtained from Dr. C. E. Ballou (Department of Biochemistry, Univ. of California at Berkeley) and maintained at 28° on Wickerham's medium or an alternate medium containing 0.1% mannose, 0.05% yeast extract, 0.5% Bacto peptone, 2% Bacto agar, plus the salts solution of Jones and Ballou (1968, 1969a).

Saccharomyces mutant strain FH4C was isolated (Montenecourt, 1968; Montenecourt *et al.*, 1973) by ultraviolet irradiation of *Saccharomyces* strain 303-67, a hybrid of *S. chevalieri*, *S. italicus*, and *S. carlsbergensis* (Winge and Roberts, 1957). The principal phenotypic advantage of the mutant is its ability to synthesize invertase when grown in high concentrations of hexose. The mutant was maintained at 4° on slants containing 1% glucose, 0.3% yeast extract, 0.5% Bacto peptone, and 1.5% Bacto agar. For large-scale production, mutant FH4C was grown in lots of 500 l. in a medium containing 4% glucose, 0.2% $(NH_4)_2SO_4$, and 1% Ardamine Z yeast extract. Poly(ethylene glycol) was added as an antifoamant. Incubation was with stirring at 28° for 18 hr. Cells were harvested by Sharples centrifugation.

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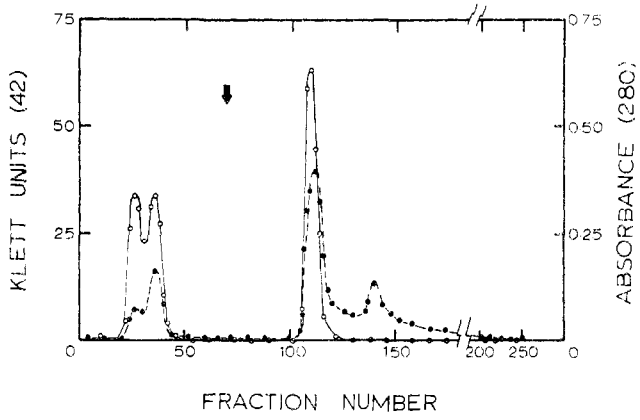

FIGURE 1: Fractionation of FH4C bulk mannan on DEAE-cellulose. Fractions were monitored for carbohydrate (○; filter 42) and 280 absorbance (●). Arrow designates start of a 200-ml 0–0.3 M NaCl gradient.

Preparation of FH4C Mannans. FH4C bulk mannan[1] was prepared by the procedure of Peat *et al.* (1961b). Purified mannan (14 g) was obtained from 2 kg of yeast cell paste.

Neutral and acidic mannans were isolated from bulk mannan by chromatography on DEAE-cellulose (DE-52, microgranular). The resin was equilibrated in 10 mM phosphate buffer (pH 7.5) then charged with bulk mannan dissolved in the same buffer. The eluate was collected dropwise with a fraction collector and monitored for carbohydrate and protein. After the neutral mannans had been collected, acidic mannan was eluted with either 1.0 M NaCl or a 0–0.3 M NaCl gradient (both prepared in 10 mM phosphate buffer). Mannan fractions were pooled, dialyzed, then lyophilized.

Mannan Core. Bulk mannan (1.05 g) was dissolved in 39 ml of 10 mM phosphate buffer (pH 6.8), containing 10^{-4} M Ca^{2+} and bovine serum albumin (50 μg/ml). α-Mannosidase (4.4 units) was added and the digest was incubated at 38° for 52 hr after which time 52% of the mannan had been converted to free mannose. The enzyme was inactivated by heating the digest (100°, 20 min). Mannose and salts were removed by dialysis; protein was precipitated from the retentate with cold 15% trichloroacetic acid. Following dialysis, mannan core was recovered by lyophilization.

Baker's yeast mannan was prepared according to Peat *et al.* (1961b). A yield of 63.4 g of purified mannan was obtained from 30 lb of wet, pressed yeast, generously supplied by Mr. E. T. Palumba, Anheuser Busch, Old Bridge, N. J.

α-Mannosidase. Arthrobacter GJM-1 was grown in the salts medium of Jones and Ballou (1968) with 1 g/l. of baker's yeast mannan as a carbon source. The supernatant fluid from a 42-hr culture was made 90% saturated with ammonium sulfate and the resulting pellet was backwashed with 60% ammonium sulfate to solubilize contaminating protein. The residual, α-mannosidase-containing pellet was dissolved in 10 mM phosphate buffer (pH 6.8) and dialyzed against the same buffer. Thirty-eight per cent of the activity was recovered with a 4.3-fold purification. It was maintained at −20° and used for subsequent experiments.

Concanavalin A, purified according to Agrawal and Goldstein (1965), was a generous gift of Dr. J. S. Tkacz, formerly of this Institute.

Purified *Escherichia coli* and calf intestinal mucosa alkaline phosphatases were obtained from Sigma.

[1] The term "bulk mannan" refers to purified FH4C mannan which has not been resolved into neutral and acidic components by DEAE chromatography.

Total carbohydrate was determined by the procedure of Dubois *et al.* (1956) with D-mannose as a standard. Reducing sugar was measured according to Nelson (1944). Protein was estimated by the method of Lowry *et al.* (1951) with bovine serum albumin as a standard. Phosphorus was determined according to Bartlett (1959) with KH_2PO_4 as a standard. A Klett–Summerson colorimeter was used for these measurements.

Descending chromatography was carried out on Whatman No. 1 chromatography paper with the following solvent systems: 1, ethyl acetate–pyridine–water (8:2:1); 2, 1-butanol–ethanol–water (3:1:1); 3, 1-butanol–ethanol–water (5:1:4, upper phase). Sugar spots were detected with alkaline $AgNO_3$ (Trevelyan *et al.*, 1950); phosphate esters were detected according to Burrows *et al.* (1952).

Titration of Mannan. Aqueous solutions of mannan were shaken briefly with 0.5–1.0 ml of cation exchanger (AG-50-X12, 20–50 mesh, H^+ form; Bio-Rad Laboratories). The supernatant mannans were recovered and transferred to small glass vials in which they were titrated at room temperature (25°) with 0.01–0.5 N NaOH delivered *via* micropipets. The pH change was monitored on a Radiometer Copenhagen pH meter standardized with buffers of pH 4 and 7.

Ouchterlony Analysis. Reactions of concanavalin A with mannans were visualized by double diffusion in gels consisting of 0.9% NaCl, 0.01% merthiolate, and 1.6% Noble agar in distilled water. (An alternate medium contained 0.85% NaCl, 1% NaN_3, and 1.3% Noble agar in 0.1 M phosphate buffer, pH 7.2.) The agar was layered on precleaned microscope slides with a pipette and wells made in the gels with an Ouchterlony template. Mannans were added *via* micropipets to the peripheral wells of the slides; the central wells were filled with concanavalin A. The gels were incubated at room temperature in humidity chambers for 12–36 hr, after which results were photographically recorded.

Selective staining of reducing disaccharides was by a modification of the procedure of Avigad *et al.* (1961). Disaccharides were spotted onto chromatography paper, then dipped into a solution containing 0.12% 2,3,5-triphenyltetrazolium chloride in chloroform + 0.5 N methanolic NaOH (4:1). The paper was heated at 85–90° (or held over a steam jet) until the sugars were stained. The intensity of the stain produced by each sugar was used as an indication of its glycosidic linkage.

Gel chromatography was performed on Sephadex G-200 (column dimensions 2 × 89 cm) with 0.1 M NaCl as an eluent, and on Bio-Gel A-1.5 m (column dimensions 1.4 × 98 cm) with 50 mM phosphate buffer (pH 7.0) (0.65 M in NaCl and 0.02% in NaN_3) as an eluent.

Results

Ionic Heterogeneity of FH4C Mannan. Purified FH4C bulk mannan was chromatographed on DEAE-cellulose and thus resolved into three component fractions (Figure 1). Two of these were not retained by the resin and are hereafter referred to as neutral mannans 1 and 2. The third fraction, which accounted for 42–49% of the original carbohydrate, required elution with NaCl and is therefore designated acidic mannan. The mannose–phosphate molar ratios of neutral mannans 1 and 2 were 94.5 and 75.1, respectively, while that of the acidic mannan was 35.5. The ratio for the unfractionated bulk mannan was intermediate (50.7). Similar fractionations of yeast cell wall mannans based on phosphate content have been reported by Thieme and Ballou (1971, 1972), Kozak and Bretthauer (1970), and Sakaguchi *et al.* (1967).

Molecular Weight of FH4C Mannan. When chromatographed on Bio-Gel A-1.5m, bulk mannan gave a very broad elution profile, an indication that the polysaccharide is polydisperse and consists of a population of components of varying molecular size (Figure 2). A small amount of the mannan was excluded from the gel, while other fragments eluted at or beyond the position of a dextran of 40,000 molecular weight. When mannan was chromatographed on Sephadex G-200, the bulk of the carbohydrate was eluted in the void volume. Thus most of the mannan molecules are >200,000 (the G-200 exclusion limit for polysaccharides). An apparent molecular weight range for FH4C mannan is from 40,000 to >300,000.

Enzymatic Susceptibility of FH4C Mannan. FH4C mannan was examined to determine its susceptibility to the bacterial α-mannosidase of Jones and Ballou (1968, 1969a,b). The enzyme, an inducible extracellular protein elaborated by *Arthrobacter* GJM-1, splits α-1,2-, α-1,3-, and α-1,6-mannosidic bonds, attacking the non-reducing termini of mannan chains and sequentially releasing single mannose residues. It has been used to characterize the mannans from several types of yeast (Gorin *et al.*, 1969; Jones and Ballou, 1969b). Table I (column 2) lists the enzymatic susceptibilities of bulk mannan, and its neutral and acidic components and that of a mannan from commercial baker's yeast (*S. cerevisiae*). With each mannan, the initial rate of enzymolysis was rapid; however, after 5–10 hr, release of mannose slowed and was nearly complete by ~24 hr. Enzymolysis was terminated by adding cold 10% trichloroacetic acid to each mannan digest. Denatured protein was removed by centrifugation and the mannan hydrolysates were neutralized, concentrated and fractionated on G-50 Sephadex (fine) with 0.1 M acetic acid as the eluent. Each mannan digest yielded a high molecular weight residue which was excluded by the gel. This material, which resists further action by the α-mannosidase, is hereafter referred to as mannan core. A second fraction recovered from each mannan was identified as mannose by chromatography in solvent system 1. Oligomannosides were absent; this is in agreement with Jones and Ballou (1968) who reported that only mannose is released by α-mannosidase.

Reactivity of Mannan Cores with Concanavalin A. Jones and Ballou (1968, 1969b) presented evidence that α-mannosidase debranches the mannan molecule and leaves a resistant, linear backbone containing predominantly α-1,6 linkages. To determine if the FH4C and baker's yeast mannans had been enzymically debranched by α-mannosidase, each core was tested for reactivity with concanavalin A, the lectin of the jack bean, *Canavalia ensiformis*. Concanavalin A precipitates branched polysaccharides leaving multiple nonreducing α-D-glucopyranosyl, α-D-mannopyranosyl, or α-D-fructofuranosyl residues as their nonreducing termini (So and Goldstein, 1968). Of all the polysaccharides reactive with this lectin, highly branched α-linked mannans are the most tightly bound. Unbranched (*i.e.*, linear) polysaccharides are not precipitated.

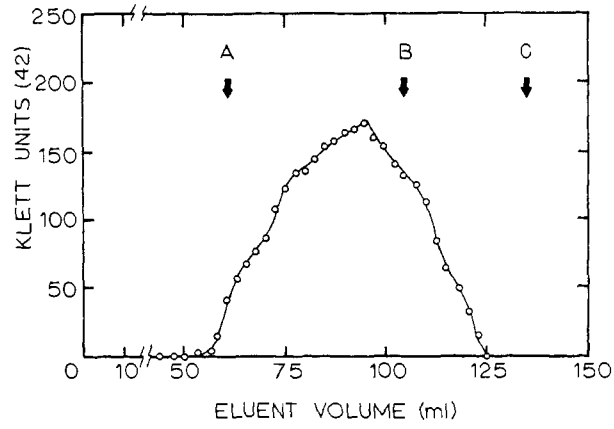

FIGURE 2: Gel filtration of FH4C bulk mannan (37.5 mg) on Bio-Gel A-1.5m (column dimensions 1.4 × 98 cm). Arrows indicate elution positions of calibration markers (A) Blue Dextran; (B) a 40,000 mol wt dextran and (C) glucose; (O) total carbohydrate.

The reactions of mannan cores with the lectin were visualized by Ouchterlony double diffusion in agar gels (Materials and Methods). Figures 3A–F show the differences between mannans and their respective cores in their reactivities with concanavalin A. Invariably, the intact parent mannan gave the more intense reaction, yielding heavier and sharper precipitin bands observable even at extreme dilutions. The cores, however, formed faint, diffuse precipitates; reactivity with the lectin was weak and could be diluted out quickly.

The intensities of reaction of the cores with concanavalin A varied inversely with the susceptibilities of the parent mannans to α-mannosidase. The core from acidic mannan (degraded only 53%) gave the heaviest bands of all the FH4C cores (3E). Bulk mannan core gave an intermediate reaction, while cores from neutral mannans 1 and 2 (>66% hydrolysis) were least reactive. The side chains of the latter may be more accessible to α-mannosidase so that "cleaner" sparsely branched cores were enzymatically produced. However, this comparison fails with the core from baker's yeast mannan (>80% hydrolyzed) which gave a stronger reaction with concanavalin A (Figure 3A,F) than the cores from neutral mannans 1 and 2 (Figure 3B,C,F).

The critical observation is that each mannan despite exhaustive enzymolysis was still capable of reacting and forming an observable precipitate with the lectin. Clearly, then, these mannans were not entirely denuded of their side chains by α-mannosidase; rather, at least some of these structures were retained in the cores. This suggests that whatever prevents further action by α-mannosidase resides in or near the oligomannosidic branches.

Nature of Mannan Phosphate. The titration studies of McLellan and Lampen (1968), Mill (1966), and Slodki (1962) demonstrated that mannan–phosphate exists in the form of phosphodiester linkages. That these bonds also occur in the

TABLE I: Susceptibilities of Mannans from Baker's Yeast and Mutant FH4C to α-Mannosidase and Correlation with Phosphate Content.

Mannan Preparation	% Hydrolysis by α-Mannosidase	Mannose:Phosphorus Molar Ratio		% Relative Enrichment in Phosphorus
		Before Hydrolysis	After Hydrolysis	
Baker's yeast mannan	81.8	196	58	240
FH4C bulk mannan	63.0	51	23	125
FH4C neutral mannan 1	66.6	95	38	146
FH4C neutral mannan 2	66.2	75	29	155
FH4C acidic mannan	48.8	36	20	81

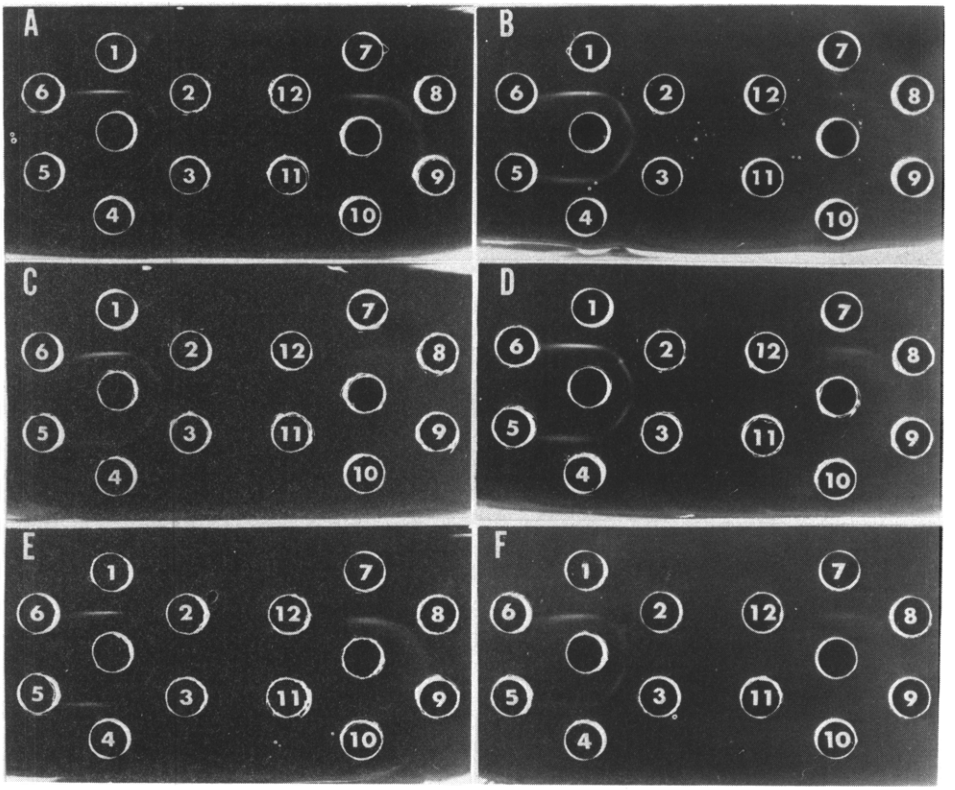

FIGURE 3: Interaction of concanavalin A with mannans and mannan cores from baker's yeast and mutant FH4C. In slides A–F, wells 1 and 7 each have 20 μg of carbohydrate either as mannan or mannan core; wells 2–6 and 8–12 contain twofold serial dilutions of the samples in well 1 and well 7, respectively: (A) wells 1–6, baker's yeast mannan; wells 7–12, baker's yeast mannan core; (B) wells 1–6, neutral mannan 1; wells 7–12, neutral mannan 1 core; (C) wells 1–6, neutral mannan 2; wells 7–12, neutral mannan 2 core; (D) wells 1–6 bulk mannan; wells 7–12, bulk mannan core; (E) wells 1–6, acidic mannan; wells 7–12, acidic mannan core; (F) wells 1–6, baker's yeast mannan core; wells 7–12, neutral mannan 1 core.

bulk mannan from mutant FH4C is shown by the titration curve in Figure 4. In the titration curve of FH4C mannan following mild acid hydrolysis (0.5 N HCl, 100°, 30 min), the inflection at pH 6.5 (the approximate pK_2 of H_3PO_4) indicates that acid treatment has cleaved phosphodiester bonds, generating phosphomonoester mannan. A titration curve with an inflection at pH ~6.5 was also obtained with unhydrolyzed mannan core, demonstrating that the latter contains some monoesterified phosphate, some generated, no doubt, by the trichloroacetic acid used to precipitate the α-mannosidase during preparation of mannan core (see Methods). After mild acid hydrolysis, the core gave a titration curve with an inflection at pH 7. However, the break in the curve was sharper than in the unhydrolyzed core, indicating that additional monoester phosphate had been unmasked by exposure to acid.

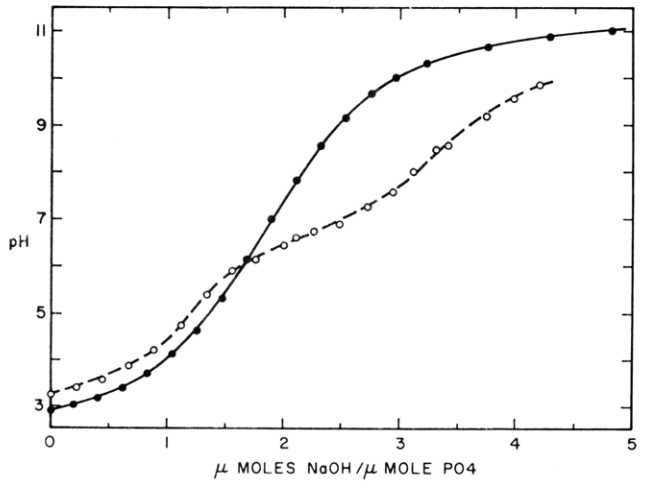

FIGURE 4: Alkaline titration curve of FH4C bulk mannan before and after mild acid hydrolysis (0.5 N HCl, 100°, 30 min): (●) unhydrolyzed; (○) acid hydrolyzed.

Constituents of Mannan Phosphodiesters. Bulk mannan and mannan core were converted into their respective phosphomonoester derivatives by heating each in 0.5 N HCl for 30 min at 100°. The hydrolysates were neutralized with NaOH and fractionated on a G-15 Sephadex column standardized with mono-, di-, and trisaccharides. Distilled water was used as the eluent. The elution profile (Figure 5) shows that mono- and disaccharides were the predominant sugars released from mannan; higher oligosaccharides were also present, but in traces. A similar elution pattern (not shown) was obtained with acid-hydrolyzed mannan core.

The carbohydrate-containing peaks from G-15 were desalted, then chromatographed on paper in solvent system 2. Two distinct sugars were detected in the material recovered from both polysaccharides. One of these chromatographed as mannose, while the second stained deeply (though very slowly) with $AgNO_3$ but corresponded to none of the marker sugars. However, its elution position from G-15 indicated a disaccharide. The latter was hydrolyzed under nitrogen (1 N HCl, 23 hr, 100°), deacidified, and chromatographed in solvent systems 1 and 2, whereupon mannose was revealed as the sole hydrolysis product. Thus, the disaccharide (designated M_2) is apparently a mannobiose.

M_2 gave a positive reaction with 2,3,5-triphenyltetrazolium chloride (Materials and Methods) and had ~74% of the molar reducing power of D-mannose. Both of these properties are inconsistent with a 1,2-linked disaccharide (Avigad *et al.*, 1961; Barker *et al.*, 1956; Côté, 1959; Swaminathan *et al.*,

1972). Furthermore, the $R_{mannose}$ of M_2 in solvent systems 2 and 3 did not correspond to published $R_{mannose}$ values of both α-1,2- and α-1,6-mannobiose (Lee and Ballou, 1965; Li, 1967). From these observations, M_2 was tentatively assigned the α-1,3-linkage (the only other known glycosidic linkage in yeast mannan) and tentatively identified as 3-*O*-α-D-mannopyranosyl-D-mannose.

Phosphate Enrichment of Phosphomonoester Mannan. The data in Table II (column 2) show that phosphate was retained in the phosphomonoester derivatives of both mannan and mannan core. Thus, after phosphodiester bond cleavage by mild acid hydrolysis, the resulting secondary phosphate remains covalently attached to the mannan. Furthermore, the decrease in carbohydrate content is consistent with a loss of oligosaccharides on cleavage of the diesters, a conclusion substantiated by Sephadex and paper chromatography.

Phosphatase Digestion of Phosphomonoester Mannan. An attempt was made to remove secondary phosphate enzymatically from phosphomonoester mannan and phosphomonoester mannan core. Each polysaccharide was dissolved in 3–4 ml of 0.1 M Tris buffer (pH 8.0) containing 100 μg/ml each of chloramphenicol and cycloheximide. The samples were transferred to dialysis bags and to each were added purified alkaline phosphatases from *E. coli* (5.0 units) and calf intestinal mucosa (20 units). The bags were sealed, immersed into flasks containing 200 ml each of the above buffer, and incubated with shaking at 30° in a Gyrotory water bath (New Brunswick Scientific Co.). Samples of unhydrolyzed bulk mannan and mannan cores were identically processed as experimental controls. After 66 hr, the mannans were recovered. The phosphatases were precipitated with trichloroacetic acid and the supernatant mannans were exhaustively dialyzed then analyzed for total phosphorus.

The low susceptibility of bulk mannan to the phosphatase (Table II) is consistent with the titration studies which indicated little or no secondary phosphate in this polymer. However, the increased susceptibility of hydrolyzed mannan confirms the acid cleavage of phosphodiester bonds to monoester phosphate. Mannan core, which initially contains considerable monoester phosphate, was more severely degraded by the phosphatases than acid-treated mannan, even though the former still has some intact phosphodiesters while the latter does not. This no doubt reflects the removal of steric barriers (*i.e.*, oligomannoside branches) during enzymolysis of mannan by α-mannosidase. The even greater susceptibility of acid-treated core again attests to cleavage of phosphodiester bonds by acid hydrolysis. The inability of the phosphatases to remove all of the phosphate from the acid-treated samples suggested that quantitative cleavage of phosphodiester bonds had not occurred; however, titrimetry revealed that this was not the case. A more likely cause is steric hindrance of these enzymes by oligomannosidic branches, a conclusion substantiated by the report of McLellan and Lampen (1968).

TABLE II: Effect of Mild Acid Hydrolysis on the Susceptibility of Bulk Mannan and Mannan Core to Alkaline Phosphatases.

Preparation Treated with Phosphatases	Mannose:Phosphorus Molar Ratio		% Release of Phosphorus
	Before Phosphatase Treatment	After Phosphatase Treatment	
Bulk mannan	50.7	54.0	6.0
Acid-hydrolyzed bulk mannan	47.4	63.5	25.4
Mannan core	24.5	37.6	34.9
Acid-hydrolyzed mannan core	22.7	43.7	48.0

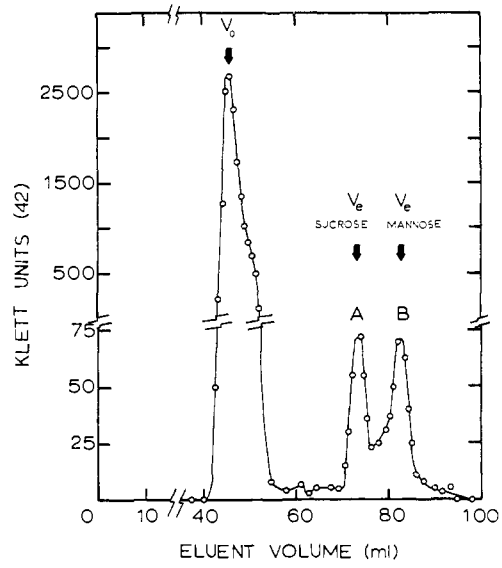

FIGURE 5: Sephadex G-15 elution profile of FH4C bulk mannan following mild acid hydrolysis (0.5 N HCl, 100°, 30 min). Fractions were monitored for carbohydrate (O) with phenol and H_2SO_4. Arrows designate elution positions of Blue Dextran (V_0), sucrose, and mannose.

Refractoriness of Yeast Mannan. Table I lists the mannose:phosphate ratios of FH4C and *S. cerevisiae* mannans before and after hydrolysis by α-mannosidase. As can be seen, all of the cores have been enriched in phosphorus as compared to the original mannans. Furthermore, the more heavily phosphorylated mannans (*i.e.*, lower mannose:phosphorus ratios) were the least susceptible to the enzyme, suggesting that the phosphate groups shield the polysaccharide from enzymolysis. A second (though less likely) steric barrier was the peptide moiety of mannan and mannan core. To assess the contribution of both protein and phosphate to the refractoriness of the polysaccharide, α-mannosidase was tested on mannan cores which had been chemically and/or enzymatically modified. These included cores which had been: (a) stripped of protein by hot alkaline hydrolysis (3.5 M NaOH, 95°, 10 hr), (b) exposed to alkaline phosphatases, or (c) deproteinated with alkali, then incubated with alkaline phosphatases. The results are summarized in Table III.

The extent of hydrolysis by α-mannosidase was essentially the same for both the unmodified and alkali-treated (*i.e.*, protein-free) cores. Thus, mannan–peptide alone is not a steric barrier to α-mannosidase. However, mannan cores which had been partially dephosphorylated were clearly more susceptible to the enzyme. This was most evident with core which had been exposed to hot alkali prior to incubation with alkaline phosphatases. The proteolytic action of the alkali was apparently of little consequence to the susceptibility of the latter core to α-mannosidase. Rather, the primary effect was the increased dephosphorylation of the core, made possible by the cleavage of phosphodiester bonds by the harsh alkaline treatment (Stewart and Ballou, 1968) with further unmasking of phosphomonoester groups. Thus, it is the bound phosphate

TABLE III: Susceptibilities of Chemically and Enzymatically Modified Mannan Cores to α-Mannosidase.

Mannan Core	% Release of PO_4 by Pretreatment with Alkaline Phosphatases	% Hydrolysis by α-Mannosidase
Unmodified mannan core (control)		19.7
Alkali-treated mannan core		22.5
Phosphatase-treated mannan core	29.7	30.5
Alkali- and phosphatase-treated mannan core	38.9	44.4

that shields the mannan molecule from hydrolysis by α-mannosidase.

This conclusion was confirmed by the following experiment. Mannan core (~21 mg) was dissolved in 2 ml of 50 mM Tris (pH 8.2) (10^{-4} M in Ca^{2+}) containing 100 μg each of chloramphenicol and cycloheximide. To this solution (A) were added 6.3 and 6.6 units, respectively, of *E. coli* and calf intestinal mucosa alkaline phosphatases. An identical core mixture (B) was prepared, but with omission of the phosphatases. A and B were incubated in a 30° water bath for 12 hr after which time 0.16 unit of α-mannosidase and another 2.1 units of each phosphatase were added to A; B received only α-mannosidase. The solutions were incubated at 30° and monitored for reducing sugar. After 52 hr, 4.2 and 4.4 units of *E. coli* and calf phosphatases, respectively, were added to solution B. After an additional 44 hr, enzymolysis was terminated by precipitating the enzymes from both digests with trichloroacetic acid. The supernatants were dialyzed and the retained cores were recovered by lyophilization. The reaction profiles in Figure 6 clearly show that the alkaline phosphatases augment the susceptibility of mannan core to α-mannosidase. After 52-hr incubation, the per cent digestion with added phosphatases (A) was 1.5 times that in the control (B). Blocking by PO_4 was verified by the observation that addition of phosphatases to B led to a further release of mannose and raised the susceptibility of the latter core to that of the pretreated material (A).

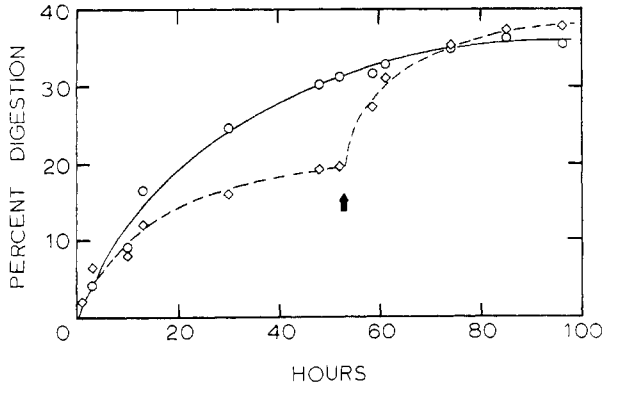

FIGURE 6: Multienzyme digestion of FH4C mannan cores. α-Mannosidase added at 0 hr: (○) preparation A, preincubated for 12 hr with alkaline phosphatases; (◇) preparation B, no alkaline phosphatases during preincubation. Arrow designates addition of *E. coli* and calf intestinal mucosa alkaline phosphatases to preparation B.

The phosphate contents of the recovered cores were essentially identical with those of the starting materials, consistent with a simultaneous release of both carbohydrate and phosphate. Alkaline titration of the combined cores indicated that little of the remaining PO_4 was monoesterified; more likely, phosphodiester linkages predominated. The latter could explain the eventual refractoriness of these cores to both α-mannosidase and alkaline phosphatases.

Discussion

Like that from other yeasts, the mannan from *Saccharomyces* mutant FH4C can be resolved on DEAE into several components of varying ionic charge. The obvious differences between the neutral and acidic fractions were the elevated protein and phosphate contents of the latter. Thus, protein and/or phosphate might have been responsible for the ionic properties of the acidic mannan. In fact, deproteinization of the latter with pronase or hot alkali released small amounts of neutral carbohydrate and a new acidic species with high affinity for DEAE. However, the neutral fragments contained little of either protein and phosphate, while the acidic species (always the major product), though poor in protein, was always heavily phosphorylated. Thus, phosphate and not protein is predominantly responsible for the ionic heterogeneity of yeast mannan.

Our molecular weight studies show that FH4C mannan consists of a heterogeneous population of molecules ranging in size from 40,000 to >300,000 daltons. Similar high molecular weight yeast mannans have been isolated by McLellan and Lampen (1968), by Eddy and Longton (1969), and by Thieme and Ballou (1972). The latter investigators have demonstrated that mannan protein crosslinks smaller mannan subunits of 25,000–35,000 molecular weight into complexes of 100,000–500,000 molecular weight. In the accompanying paper (Colonna and Lampen, 1974) we present evidence in support of their hypothesis.

By titrimetry, it was shown that the phosphate in FH4C mannan is present in the form of phosphodiester bonds. These linkages have been demonstrated in mannans from *Saccharomyces cerevisiae* (Mill, 1966), *S. carlsbergensis* (McLellan and Lampen, 1968), *Hansenula capsulata* (Slodki, 1962), and *Kloeckera brevis* (Thieme and Ballou, 1971). Thieme and Ballou (1971) showed that mild acid hydrolysis of *K. brevis* phosphodiester mannan released mannose in an amount equal to the total mannan phosphate; from *S. cerevisiae* mannan, acid hydrolysis released equimolar quantities of mannose and mannobiose. They concluded that in mannan, phosphodiester linkages bridge mono- and/or disaccharide residues to high molecular weight mannan chains. In our studies, mild acid hydrolysis of FH4C mannan released both mannose and mannobiose (the latter tentatively identified as 3-*O*-α-mannopyranosyl-D-mannose). Therefore, in agreement with Thieme and Ballou (1971), mono- and disaccharides represent one terminus of the phosphodiester bonds in FH4C mannan. In this respect the mannan from mutant FH4C (derived from *S. chevalieri*, *S. italicus*, and *S. carlsbergensis*) resembles that of *S. cerevisiae*.

Several investigators have identified mannose 6-phosphate as a constituent of yeast mannans (Slodki, 1962; McLellan and Lampen, 1968; Sentandreu and Northcote, 1968). This ester has also been found in the mannan from mutant FH4C (Colonna, 1972). Clearly, then, one end of the phosphodiester linkage in mannan involves C-6 of polymeric mannose residues. However, until recently, the other terminus of this

phosphate bridge has eluded unambiguous identification. The acid lability of this second linkage point has been well documented (Mill, 1966; McLellan and Lampen, 1968; Slodki, 1962; Thieme and Ballou, 1971). Favorably received was Slodki's hypothesis that, in mannan, a phosphate group links the anomeric (*i.e.*, C-1) hydroxyl of a mannose unit to C-6 of an adjacent mannose residue. Recently, Thieme and Ballou (1971), using high-resolution proton magnetic resonance spectroscopy, showed that in *K. brevis* phosphodiester mannan, mannose is linked to phosphate through C-1. Their finding was confirmed by the immunochemical studies of Raschke and Ballou (1971). Thus, mannose 1-phosphate represents the acid-labile moiety of mannan phosphodiester bonds. In *K. brevis* mannan, these bonds join single mannose residues to C-6 of the subterminal mannose in a mannobiose side chain (Thieme and Ballou, 1971). Presumably, in both *S. cerevisiae* and FH4C mannans, a similar arrangement exists whereby mannose and/or mannobiose residues are linked through phosphate to C-6 of a mannose unit in di- or trisaccharide branches originating from the α-1,6-linked backbone.

Each of the mannans examined showed varying susceptibility to hydrolysis by α-mannosidase. This is consistent with the variations in enzymatic hydrolysis observed by Jones and Ballou (1969b) with mannans from other yeasts and indicates that some yeast mannans may be structurally more complex than others. Furthermore, the range of susceptibilities observed among the FH4C mannan fractions implies that structural variation exists even in the mannan from a single yeast strain.

Neither baker's yeast mannan nor any of the mannans from mutant FH4C were completely hydrolyzed by α-mannosidase. Such refractoriness of yeast mannans to enzymatic hydrolysis was first reported by Jones and Ballou (1968, 1969b). In our investigation, refractoriness was not caused by inactivation of α-mannosidase during the prolonged hydrolysis, since addition of fresh enzyme was ineffectual. Rather, something inherent in the mannan molecule prevented further action by the enzyme.

Jones and Ballou (1968, 1969b) reported that baker's yeast mannan was degraded $\sim$65% by α-mannosidase. Since α-1,2 and α-1,3 linkages account for $\sim$65% of the total mannosidic bonds (Peat *et al.*, 1961a), and since they occur in the mannan side chains, these investigators proposed that the mannosidase debranches the mannan molecule, leaving a linear "core" containing predominantly α-1,6 linkages. Their results from acetolysis of baker's yeast mannan core support this proposal.

In our studies, the per cent hydrolysis of baker's yeast mannan ($\sim$82%) exceeded the usual percentage (65%) of mannose residues joined by α-1,2 and α-1,3 bonds (Peat *et al.*, 1961a). Therefore, the α-mannosidase must attack at least some of the α-1,6-linked mannose residues which comprise the rest of the polysaccharide. This is substantiated by the report of Tkacz *et al.* (1972) that synthetic poly[α-(1,6)-D-mannopyranose] is susceptible to hydrolysis by the *Arthrobacter* α-mannosidase. Therefore, the refractoriness of mannan is not due to an inability of the enzyme to break α-1,6-mannosidic bonds when these occur in large polymers; rather, it appears that mannan core has a more complex structure than that of an α-1,6-linked linear polymannose chain. This was demonstrated by the observation that each mannan (including that from baker's yeast), is spite of exhaustive hydrolysis by α-mannosidase, could still react and form a precipitate with concanavalin A. It has been shown that a linear polymer of mannose residues joined by α-1,6 bonds is incapable of precipitating with concanavalin A (Tkacz, 1971). Thus, in contrast with the findings of Jones and Ballou (1969b), mannans from baker's yeast and mutant FH4C are not completely debranched by α-mannosidase; rather, at least some of these structures are retained in the cores, suggesting that whatever is responsible for the refractoriness of mannan residues is in or near the mannan side chains.

Jones and Ballou (1969b) observed that phosphate-poor mannans were more susceptible to enzymatic hydrolysis than more heavily phosphorylated mannans and postulated that bound phosphate somehow impedes the action of α-mannosidase. Their observation is in complete agreement with our data on enzymolysis of mannans from *S. cerevisiae* and mutant FH4C (Table III). Our degradative studies on FH4C mannan and mannan core using chemical and enzymatic procedures show that mannan–peptide alone does not contribute to the refractoriness of mannan. However, substantial increases in the susceptibility of mannan core to α-mannosidase were observed in the presence of/or following incubation with alkaline phosphatases. From these observations, we conclude that mannan–phosphate sterically shields the mannan molecule from total enzymatic hydrolysis; removal of phosphate exposes potentially susceptible mannosidic bonds, resulting in further depolymerization by α-mannosidase.

Similarly, the susceptibility of mannan–phosphate to alkaline phosphatases was related to the size of the mannan polymer (*e.g.*, compare acid-hydrolyzed bulk mannan with acid-hydrolyzed mannan core, Table II). McLellan and Lampen (1968) observed that *E. coli* alkaline phosphatase failed to release inorganic phosphate from phosphomonoester mannan from *S. carlsbergensis*. Their conclusion, which is consistent with our findings, was that the polysaccharide impeded the action of the phosphatase. Thus, a reciprocal relationship exists wherein the mannan is partially shielded from α-mannosidase by bound phosphate while the latter is likewise sterically protected from alkaline phosphatases by the highly branched mannan structure.

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Structure of the Mannan from *Saccharomyces* Strain FH4C, a Mutant Constitutive for Invertase Biosynthesis. II. Protein Moiety and Components of the Carbohydrate–Peptide Bonds†

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ABSTRACT: The cell wall mannan of *Saccharomyces* mutant FH4C has been extracted and separated by ion-exchange chromatography into one acidic and two neutral fractions. The protein moieties of these preparations are rich in serine and threonine which carry oligomannosides of DP 1–4 that are released by dilute alkali. These comprise ~11% of the total mannan and their order of abundance is $M_2 > M_1 \approx M_3 > M_4$. The oligosaccharides are susceptible to enzymolysis by a bacterial α-mannosidase with resultant enrichment of the monosaccharide fraction; however, the mannose–*O*-Ser (Thr) bond is not attacked. The amino acid composition of FH4C mannan protein is similar to those of other mannan peptides. Asp, Glu, Ala, Ser, and Thr comprise >65% of the total protein; over 70% of each of the latter two acids possess attached oligomannosides. FH4C mannan contains 0.5–0.8% of glucosamine. Mannans deproteinated with hot alkali lack this sugar, suggesting that it became exposed and subsequently destroyed during the proteolysis. Thus, glucosamine may represent the reducing terminus of individual mannan chains and may join these to mannan peptide by *N*-glycosidic bonds to asparagine. The molar excess of aspartic acid in all mannans examined strengthens the latter possibility. The mannose : glucosamine ratio of FH4C mannan indicates that individual mannan chains have a molecular weight range of 19,000–37,000. Mannan peptide appears to cross-link these mannan chains to form macromolecules >300,000.

Yeasts have cell walls comprised predominantly of glucan and mannan. The former is a sparsely branched polymer of glucose monomers joined by β-1,3 linkages; some β-1,6 bonds are also present. In contrast, mannan is a highly branched α-linked mannose polymer containing 1,2-, 1,3-, and 1,6-mannosidic bonds (50:15:35 in the usual baker's yeast).

Mannan contains small amounts of protein (4–8% by weight; Eddy, 1958; Sentandreu and Northcote, 1968) which is postulated (Thieme and Ballou, 1972) to cross-link mannan chains, thereby increasing the rigidity of the cell wall and providing a matrix for extracytoplasmic enzymes, *e.g.*, acid phosphatase or invertase.

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