A Study of the Phosphate Linkages in Phosphomannan in Cell Walls of *Saccharomyces cerevisiae*

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1. The phosphomannan of *Saccharomyces cerevisiae* was released by Pronase digestion of cell walls and isolated by chromatography on DEAE-cellulose or by precipitation with borate–Cetavlon solutions. Mannose and phosphorus were present in the molar ratio 18:1 and the phosphate groups were in the diester form. 2. Hydrolysis with acid gave mannose 6-phosphate. Under mild acid conditions (autohydrolysis) the phosphate groups were converted into the monoester form, mannose was released and the molecular size of the phosphomannan was substantially decreased. 3. Hydrolysis with alkali also gave a monoester phosphate and a similar decrease in molecular weight. Under mild alkaline conditions the serine and threonine content of the phosphomannan was decreased by about 80%. The phosphate content was not altered. 4. Treatment with 40% (v/v) HF removed 70% of the phosphorus from the phosphomannan with no detectable decrease in molecular weight. 5. Periodate oxidation gave an oxophosphomannan from which 80% of the phosphorus was eliminated under mild alkaline conditions. 6. The properties of the phosphomannan are consistent with a structure in which the phosphate groups are located on the outside of the molecule and link C-1 of a terminal mannose unit with C-6 of another mannose unit, which is in turn attached to the polysaccharide backbone of the molecule. 7. The implications of this structure are discussed in relation to flocculation.

Since their discovery as extracellular products of the yeast *Hansenula holstii* (Anderson *et al.*, 1960), phosphomannans have also been shown to occur as components of the walls of a number of yeasts. The phosphorylated mannan produced by *H. holstii* contained D-mannose and phosphate in the molar ratio 5:1 (Jeanes *et al.*, 1961). D-Mannose 6-phosphate was identified as the only phosphorylated product in acid hydrolysates of the phosphomannan (Slodki, 1962). Evidence also suggested that phosphate occurred in diester linkage between C-6 of one mannose unit and the C-1 hemiacetal position of another. Jeanes & Watson (1962) have shown that phosphate diester linkages of this type do not yield P_i when heated with alkali or on mild treatment with acid. However, the phosphate ester linkages are activated by periodate oxidation, which results in their being in β -aldehyde positions and liable to elimination by dilute alkali, giving P_i as the product. It was also demonstrated that mannose 6-phosphate was not present in acidic hydrolysates of the periodate-oxidation product of

extracellular phosphomannans, thus indicating the destruction of mannose 6-phosphate residues by periodate treatment. Jeanes & Watson (1962) concluded that these observations supported the hypothesis that diester phosphate groups were held between C-6 of mannose units otherwise linked through C-1, and C-1 of mannose units which in turn had a mannosidic attachment at C-2. A structure was proposed in which the phosphate groups cross-linked oligosaccharide chains containing 1,2- and 1,3-linked mannose units. Valuable structural information can thus be obtained by studying the action of acid and alkali on phosphomannans and on the periodate-oxidation products derived from them. Such preliminary studies on the phosphomannan isolated from cell walls of *Saccharomyces cerevisiae* have indicated (Cawley & Letters, 1968) the presence of α -1,6-phosphodiester bonds similar to those occurring in the extracellular phosphomannans. We now report a detailed investigation of the phosphorus-containing compounds that are released from yeast cell wall by Pronase digestion and of the effects on them of treatment with acid and with alkali. Special interest has

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been attached to the location of the phosphate groups in cell-wall phosphomannan and the nature of the phosphodiester linkages.

Materials and Methods

Materials

The yeast used in this study was a strain of *Saccharomyces cerevisiae* (Guinness strain no. 1406) with class II flocculating properties (Gilliland, 1957). Whatman DEAE-cellulose was obtained from H. Reeve Angel and Co. Ltd., London E.C.4, U.K. Ion-exchange resins (Bio-Rad) and Pronase (grade B) were purchased from Calbiochem, Los Angeles, Calif., U.S.A. Calf intestinal phosphomonoesterase and D-mannose 6-phosphate were supplied by Sigma Chemical Co., St. Louis, Mo., U.S.A.

Growth of the yeast

The yeast was grown on a basal medium with the following composition (per litre of water): $(\text{NH}_4)_2\text{SO}_4$, 2.0g; KH_2PO_4 , 2.0g; CaCl_2 , 0.3g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5g; peptone, 5.0g; yeast extract, 5.0g; glucose, 75g. Medium (100ml in a 250ml conical flask) was seeded with yeast from an agar slope and placed in a Gyrotary incubator at 25°C (New Brunswick Scientific Co.; model G25). After 24h the contents of this flask were transferred aseptically to fresh medium (1 litre) in a 2-litre conical flask, which was placed in the incubator for 24h at 270 oscillations/min. Cells were harvested by centrifugation for 15min at 300g and dried between sheets of filter paper. The yield was 25g pressed wet wt./litre.

Preparation of cell walls

The wet pressed cells (10g) were mixed with water (10ml) and glass beads (10g; 0.25–0.30mm) and the mixture was shaken in a Braun disintegrator (Merkenschlager *et al.*, 1957) for 1–1½min at 4000 rev./min at 0°C. The course of the breakage was followed by microscopic examination. Broken cells had a punctured appearance and possessed the characteristic shape of the intact organism, although containing no intracellular material. The suspension of broken cells was separated from the glass beads by decantation and centrifuged at 150g for 15min at 4°C to give two distinct layers. The top layer was mainly cell debris and was removed by careful decantation. The bottom layer was suspended in sucrose–phosphate buffer (0.1M-sucrose–0.1M- KH_2PO_4 adjusted to pH 7.4 with 2M-NaOH), containing EDTA (2%, w/v). The suspension was

centrifuged at 150g for 15min and the supernatant was decanted. The broken cells were resuspended in buffer and the process was repeated five times. The cell walls were washed free from buffer and extracted with 2 × 100ml of boiling ethanol–water (4:1, v/v) for 15min. The defatted cell walls were washed with acetone and ether and dried (yield, 200mg).

Pronase digestion of cell walls

Cell wall (250mg) was suspended in water (10ml) and treated with Pronase (5mg) at 37°C. Samples (0.1ml) of the mixture were removed at intervals, made up to 1ml with water and centrifuged, and the supernatant was analysed for total carbohydrate and, after hydrolysis, for glucose and mannose. Cell-wall material that was not solubilized by Pronase digestion was isolated by centrifugation.

Treatment of phosphomannan

Isolation. Cell walls (1g) were suspended in water (50ml) and stirred for 15min. Pronase (25mg) was added to this suspension and the mixture was stirred for a further 15min at room temperature and then incubated for 18h at 37°C. The digest was centrifuged at 1500g and the phosphomannan was isolated from the supernatant by one of the following procedures.

(a) The supernatant was applied directly to a column (45cm × 2cm diam.) of DEAE-cellulose (floc type; acetate form). The column was eluted with water (100ml), which removed mainly glucan material. Elution with 0.2M-ammonium acetate (150ml) removed the phosphomannan. This eluate was concentrated to 10ml and dialysed against water for 48h. The non-diffusible fraction was concentrated by evaporation *in vacuo* to 5ml and passed through a column (10cm × 1cm diam.) of cation-exchange resin (Bio-Rad AG 50W; X2; 50–100 mesh; H^+ form) to remove protein. The column was eluted with water (100ml) and the eluate was adjusted to pH 7 with 0.1M-LiOH and concentrated by evaporation *in vacuo* to 2ml. Addition of acetone–ethanol (10ml; 1:1, v/v) precipitated the phosphomannan as the lithium salt. The precipitate was washed three times with acetone, three times with ether and dried (yield, 200mg).

(b) Sodium borate (20ml of a 5%, w/v, soln.) and Cetavlon (cetyltrimethylammonium bromide; 20ml of a 5%, w/v, soln.) were added to the supernatant (50ml). The precipitate was washed with water (1ml) and then dissolved in acetic acid (2ml) and ethanol (30ml) was added. The resulting precipitate was washed successively with ethanol, acetone and ether, and dried to give the free acid form of the phosphomannan (yield, 220mg).

Electrophoresis. The phosphomannan was sub-

jected to electrophoresis on Whatman no. 1 paper at pH 5 for 6 h at 30 V/cm.

Amino acids present in acid hydrolysates of the preparations were separated by high-voltage electrophoresis on silicic acid-impregnated paper (Chromedia SG81; H. Reeve Angel and Co. Ltd.) a buffer at pH 2 (10%, v/v, acetic acid adjusted with 2M-NH₃). A potential gradient of 100 V/cm was applied for 45 min.

Gel filtration. An aqueous solution of phosphomannan (5 mg/ml) was applied to a column (40 cm × 1.3 cm diam.) of an agarose gel (Bio-Gel A-15M; 100–200 mesh) and the column was eluted with water containing 0.02% NaN₃. Fractions (0.75 ml) were analysed for total carbohydrate by the phenol-H₂SO₄ procedure. The retention volumes were measured from the point of application of the sample to the centre of each peak, which was readily determined because of the almost perfect Gaussian distributions obtained from all the standard polysaccharides (from Pharmacia, Uppsala, Sweden; see also Bathgate, 1970) and phosphomannan fractions.

Acid hydrolysis. (i) Phosphomannan (30 mg) was passed through a column (5 ml) of Bio-Rad AG 50 (H⁺ form) resin. The eluate was evaporated to dryness and the residue dissolved in the minimum amount of water. The solution was heated at 100°C for 15 min, cooled and made up to 10 ml with water.

(ii) Phosphomannan (50 mg) was treated with 40% (v/v) HF (1 ml) in a polythene tube for 1 h at room temperature. The sample was cooled to -40°C and sufficient LiOH was added to neutralize the HF. When the sample had attained 0°C its pH was adjusted to 7 with Li₂CO₃. The insoluble LiF was removed by centrifugation and washed four times with water. The supernatants were combined and concentrated by evaporation *in vacuo* to 2 ml. Equal quantities of acid-washed charcoal (Darco 60) and Celite (grade 535) were made into a slurry in 0.01M-formic acid. The slurry was used to form a column (4 cm × 1.2 cm), which was washed with water until neutral. The concentrated supernatant was applied to this column, from which water (50 ml) eluted material (1.45 mg) that was identified as mannose by paper chromatography. Elution with aq. 30% (v/v) ethanol did not remove any additional carbohydrate material from the column. The degradation product from treatment with HF was isolated by acetone precipitation. The white precipitate was washed with acetone (three times) and ether (twice), and dried (yield, 38 mg).

(iii) Phosphomannan (300 mg) was dissolved in 2M-HCl (20 ml) and heated in a sealed tube at 100°C for 2 h. Water (10 ml) was added and the HCl was removed by extraction with a chloroform solution of tri-*n*-octylamine, as described by Sentandreu & Northcote (1968). The aqueous layer was subjected to preparative paper chromatography in solvent A.

Examination of phosphate (I) produced by acid hydrolysis

A tentative characterization was made by applying the following procedures. (i) Treatment with phosphomonoesterase at 37°C for 18 h. (ii) Conversion into the pyridinium salt by repeated evaporation in pyridine; the product was dissolved in pyridine (1 ml) containing triethylamine (0.1 ml). Water (0.1 ml) and dicyclohexylcarbodi-imide (10 mg) were added and the reaction mixture was left at room temperature for 18 h (Piras & Cabib, 1963). Water (1 ml) was added and the mixture extracted six times with ether. The reaction product was chromatographed in solvent A, before and after treatment with 0.1M-HCl at room temperature for 1 h (Brown *et al.*, 1952). (iii) The reaction was also carried out by using dicyclohexylcarbodi-imide in anhydrous methanol and in the presence of triethylamine (Piras & Cabib, 1963). (iv) Titration with 2.5mM-NaOH in the absence of CO₂.

Hydrolysis of the phosphomannan with alkali

(a) The phosphomannan (50 mg) was treated with 0.1M-KOH (10 ml) at 20°C for 24 h. The sample was neutralized with 1M-acetic acid, dialysed and the dialysis residue evaporated to dryness.

(b) Phosphomannan (30 mg) was dissolved in 1M-NaOH (5 ml) and heated for 1 h at 100°C in a polythene tube. The sample was passed through a cation-exchange resin (Bio-Rad; H⁺ form) and the eluate titrated with 2.5mM-NaOH in the absence of CO₂. The product of hydrolysis in alkali was isolated by adding sodium borate (5 ml of a 5% soln.) and Cetavlon (5 ml of 5% soln.) to an alkaline hydrolysate of the phosphomannan (200 mg in 5 ml of 1M-NaOH at 100°C for 1 h). The precipitate was converted into the free acid form by addition of acetic acid (1 ml) and ethanol (30 ml). The resulting precipitate was washed successively with ethanol, acetone and ether and dried (yield 120 mg).

Periodate oxidation

The phosphomannan (0.1%, w/v) was oxidized by sodium metaperiodate (7.4mM) buffered in 0.5M-ammonium acetate buffer, pH 5 (100 ml), and in 100 ml of unbuffered solution, in the dark at 4°C. These conditions limit over-oxidation (Jeanes & Watson, 1962). Periodate uptake was measured, on a portion (5 ml) from the buffered reaction, by the method of Malaprade (1928). Formic acid was measured on a portion (5 ml) from the unbuffered solution by adding ethylene glycol (1 ml) and titrating to the first phenolphthalein end-point with 0.5mM-Ba(OH)₂. The titrations were corrected for a reagent blank.

Elimination of P_i from periodate-oxidized phosphomannan was also studied. An unbuffered solution

of the phosphomannan at pH 5 (0.1%, w/v) was oxidized with sodium metaperiodate (7.4 mM) at 4°C for 72 h in the dark. Ethylene glycol (1 ml) was added. To a sample (6 ml), ethylene glycol (1 ml) was added and the mixture was dialysed for 48 h at 2°C. The sample was adjusted to pH 11.5 with 0.1 M-KOH and left at room temperature for 2 h. It was then made up to 10 ml with water and portions (0.5 ml) were removed for determination of P_i (Shin, 1962).

Analytical methods

Cell walls and preparations therefrom were hydrolysed at 100°C for 18 h in 1 M- H_2SO_4 for carbohydrate determinations, for 16 h in 6 M-HCl for amino acid determinations and for 16 h in 3 M-HCl for amino sugar determinations.

Phosphorus (Shin, 1962) and nitrogen (micro-Kjeldahl method with selenium catalyst; Letters, 1964) were determined by standard methods. The phosphorus contents of spots located on paper chromatograms were determined as described previously (Letters, 1964). Optical rotations were measured at 16°C with a Perkin-Elmer 141 automatic polarimeter. The phenol- H_2SO_4 (Dubois *et al.*, 1956) and tryptophan-borosulphuric acid (Badin *et al.*, 1953) reactions were used for carbohydrate determinations. Amino acid and amino sugar analyses (by Dr. K. Morgan) were carried out essentially as described by Sentandreu & Northcote (1968).

Paper chromatography

Unless otherwise indicated this was carried out on Whatman no. 1 paper by the descending technique for 18 h. The following solvent systems were used: A, propan-2-ol-aq. NH_3 (sp.gr. 0.88)–water (6:3:1, by vol.) for separating phosphate esters; B, ethyl acetate–pyridine–water (10:4:3, by vol.) for separating mannose and glucose and their oligosaccharides; C, phenol–water (3:1, v/v; ascending); D, butan-1-ol–acetic acid–water (6:2:3, by vol.; ascending) for separating amino acids. Compounds were detected with the periodate–Schiff reagent for α -glycols (Baddiley *et al.*, 1956), the $AgNO_3$ –alkali method for neutral sugars and polyols (Trevelyan *et al.*, 1950), the molybdate reagent for phosphoric esters (Hanes & Isherwood, 1949) and the ninhydrin reagent (0.2%, w/v, in acetone) for amino acids.

Results

Pronase digestion of the cell walls

The cell-wall preparation contained 84–87% of carbohydrate (allowing for decomposition of glucose and mannose during prolonged hydrolysis), 0.35% of P and 1.1% of N. Of the carbohydrate, glucan accounted for 51% and mannose for 49%. Pronase digestion resulted in the isolation of two fractions, the phosphomannan (carbohydrate 92%, N 0.8%, P 1.0%, with a mannose:glucose ratio of 1:0.05)

Table 1. Amino acid recoveries after hydrolysis of cell-wall samples with 6 M-HCl at 100°C for 16 h

The values given are in μ mol recovered from 50 mg samples. Glucosamine was determined after hydrolysis for 16 h with 3 M-HCl.

Amino acid residue	Cell wall	Phosphomannan*	Cell-wall residue after Pronase digestion	Phosphomannan + 1 M-NaOH at 100°C for 1 h
Asp	2.7	2.8	0.76	1.62
Thr	3.5	3.8	0.78	0.83
Ser	3.2	3.6	0.65	1.05
Glu	2.8	2.8	0.72	1.92
Pro	1.4	1.4	0.28	1.57
Gly	1.55	1.53	0.86	0.69
Ala	3.2	3.13	1.01	2.65
Val	1.9	1.86	0.50	1.32
Ile	1.25	0.56	0.46	0.46
Leu	1.3	0.33	0.48	0.28
Tyr	0.5	0.14	0.07	0.13
Phe	0.75	0.07	0.21	0
Trp	0.4	0	0	0
Lys	1.25	0.33	0.30	0.23
His	0.3	0.16	0.13	0.08
Arg	0.45	0	0.14	0
GlcN	3.0	0.20	0.95	0.34

* Isolated by precipitation with Cetavlon (see the Materials and Methods section).

and a residue (carbohydrate 99%, N 0.2%, P 0.2%). Amino acid analyses of the cell wall, the residue after Pronase digestion, and the isolated phosphomannan before and after treatment with 1 M-NaOH, are shown in Table 1.

The course of Pronase digestion was followed by determination of total carbohydrate and the mannose/glucose ratio of the supernatant (Fig. 1). After 18h, when 50% of the cell-wall carbohydrate was solubilized, phosphomannan preparations were isolated in approximately equal yields by the two procedures described. Both had the same composition (given above) and $[\alpha]_D^{16} +82^\circ$. Titration of the free acid form of the cell-wall mannan showed the presence of phosphodiester groups (diester P:monoester P ratio 20:1). The borate-Cetavlon procedure, which is a modification of the method published by Barker *et al.* (1957), was normally employed for the isolation of phosphomannan. Streaks rather than discrete bands were obtained on electrophoresis. No further purification could be achieved by gel filtration on agarose beads.

Gel-filtration studies

The retention volumes plotted against weight-average molecular weight ($\bar{M}_w$) and number-average molecular weight ($\bar{M}_n$) are shown in Fig. 2, for a range of standard polysaccharides and for the phos-

Fig. 1. Solubilization of carbohydrate from walls by treatment with Pronase

Walls were suspended in water and Pronase was added. The suspension was centrifuged and the amount of carbohydrate (●) and the mannose/glucose ratio (■) in the supernatant were measured at intervals. Details are given in the text.

phomannan as isolated (arrow 2), or treated with 40% HF for 2h (arrow 2), or allowed to autohydrolyse (arrow 3) or treated with 1 M-NaOH at 100°C for 1h (arrow 4). The results show the phosphomannan to have a molecular weight ($\bar{M}_n$) in excess of 200000, which was not detectably altered by HF dephosphorylation, but was decreased to around 200000 by autohydrolysis or mild alkaline hydrolysis.

Acid hydrolysis of the phosphomannan

Paper chromatography of the 2M-HCl hydrolysate gave two broad bands showing positive carbohydrate reactions, only one of which also gave a positive molybdate reaction. This latter band was eluted and rechromatographed in the same solvent. The major component (phosphate I) had the same R_F value as mannose 6-phosphate (0.23 in solvent A). The molar ratio mannose:phosphate was 1:1. It was completely degraded by treatment with phosphomonoesterase at

Fig. 2. Gel filtration of polysaccharides

Logarithmic plots are shown of number-average mol.wt. ($\bar{M}_n$; ■) and weight-average mol.wt. ($\bar{M}_w$; ●) versus retention volume of Bio-Gel A-15 M. (1) Dextran 2000 ($\bar{M}_n > 2000000$); (2) wall phosphomannan before and after treatment with 40% HF for 2h; (3) autohydrolysate of wall phosphomannan; (4) wall phosphomannan treated with 1 M-NaOH at 100°C for 1h; (5) dextran 500 ($\bar{M}_n = 212000$; $\bar{M}_w = 516000$); (6) dextran 250 ($\bar{M}_n = 121000$; $\bar{M}_w = 240000$); (7) dextran 150 ($\bar{M}_n = 86000$; $\bar{M}_w = 154000$); (8) dextran 110 ($\bar{M}_n = 113000$; $\bar{M}_w = 80000$); (9) Sigma dextran ($\bar{M}_w = 72600$); (10) dextran 40 ($\bar{M}_n = 25700$; $\bar{M}_w = 41800$). Details are given in the text.

37°C for 18h, producing free mannose. Treatment with dicyclohexylcarbodi-imide in aq. pyridine containing triethylamine gave a product (IV) with the same R_F value (0.55) as a sample of mannose 4,6-hydrogen phosphate obtained by treating authentic mannose 6-phosphate with this reagent. The cyclic phosphate (IV) was not degraded by 0.1M-HCl at room temperature for 1h, conditions that degrade five-membered cyclic phosphates (Brown *et al.*, 1952) but which we have found do not degrade mannose, its 4,6-hydrogen phosphate or methyl mannoside 4,6-hydrogen phosphate (V). The latter compound (R_F 0.68) was obtained by treating an authentic sample of methyl α -D-mannoside 6-phosphate (II, R_F 0.41) (Cawley & Letters, 1971) with dicyclohexylcarbodi-imide under the conditions described above. A similar treatment of methyl α -D-mannoside 4-phosphate (III, R_F 0.46) (Cawley & Letters, 1971) gave a cyclic phosphate (VI, R_F 0.70), which was readily degraded in 0.1M-HCl at room temperature, presumably to a mixture of 3- and 4-phosphate (VII). When triethylamine was omitted from the dicyclohexylcarbodi-imide reaction mixture we found that phosphate (I), mannose 6-phosphate and methyl α -D-mannoside 6-phosphate yielded the same cyclic phosphates as were obtained by reaction in the presence of triethylamine. Methyl α -D-mannoside 4-phosphate yielded an *N*-phosphorylurea (R_F 0.95–0.98) in the absence of triethylamine.

On reaction with dicyclohexylcarbodi-imide in anhydrous methanol and in the presence of triethylamine (Piras & Cabib, 1963), mannose 6-phosphate and phosphate (I) gave products that were identical on paper chromatography and were not hydrolysed by 0.1M-HCl at 100°C for 15 min. The product from methyl α -D-mannoside 6-phosphate was also stable under these conditions, but the product from methyl α -D-mannoside 4-phosphate was readily degraded.

Autohydrolysis resulted in a 72% yield of a partly degraded product (molecular weight approx. 200000) with an increased phosphate content (1.3%), all of which was shown by titration to be in the monoester form. This was not degraded by phosphomonoesterase under conditions that completely degraded glucose 6-phosphate. It was, however, dephosphorylated by treatment with HF to the same extent (70%) as glucose 6-phosphate treated similarly. During autohydrolysis 1 mol of mannose was released per mol of diester phosphate hydrolysed. Mannose di- and tri-saccharides were also detected in this autohydrolysis.

Phosphomannan (50mg) treated with HF was progressively dephosphorylated over 2h, yielding 38mg of a product, the phosphate content of which was decreased to 0.3% without apparent change in molecular size (Fig. 2). Mannose, to the extent of 0.75mol/mol of phosphate removed, was released during the treatment. Chromatographic analysis

failed to show the presence of mannose oligosaccharides.

Alkaline hydrolysis of the phosphomannan

Hydrolysis with 0.1M-KOH at 20°C for 24h removed 10–20% of the carbohydrate in the form of mannose and mannose oligosaccharides (di-, tri- and possibly tetra-saccharide), which were detected by gel filtration (Bio-Gel P-2) and by paper chromatography. The non-diffusible residue had a greater retention volume on agarose gel (molecular weight approx. 200000), contained half as much nitrogen (0.5%) and less than one-third of the amounts of serine and threonine present in the phosphomannan. It contained 1.3% of P, shown by titration to be entirely in the monoester form, but which, like the autohydrolytic product, was unaffected by phosphomonoesterase. Treatment with 40% HF progressively decreased the phosphate content to 0.3% during 2h incubation, which did not alter the gel-filtration characteristics of the molecule or release significant amounts of mannose.

Periodate oxidation of the phosphomannan

Periodate consumption was 0.45mol/mol of mannose, and 0.095mol of formic acid/mol of mannose was produced, in the reaction during 1h, at which time the reaction was shown to be complete, after dialysis and incubation at pH11.5 for 2h at room temperature.

Of the total phosphate in the original phosphomannan 60% was present as P_i . When the oxidation mixture was maintained at pH13.5 for 5h 80% of the total phosphate in the sample was present as P_i . Treatment of the phosphomannan with 0.1M-KOH for 24h at room temperature did not release P_i .

Discussion

The action of proteolytic enzymes, obtained from species of *Streptomyces*, on isolated yeast cell walls has been described by Nickerson (1963), who found that degradation of isolated cell walls by keratinase (produced by *Streptomyces fradiae*) was complete in 42 min and degradation by Pronase (from *Streptomyces griseus*) was complete within 72h at 25°C. We have found that isolated cell walls of *Sacc. cerevisiae* were solubilized to the extent of about 50% by Pronase digestion for 18h at 37°C. The mannose-containing compounds released by Pronase digestion accounted for 30–35% of the isolated cell wall. The additional carbohydrate (10–15% of total cell-wall material) released by Pronase digestion of cell walls was a glucan that does not contain phosphorus and that was readily eluted from DEAE-cellulose by water.

When isolated cell walls were digested with Pronase

the phosphomannan was released quickly and then glucan material was solubilized at a very slow rate. This method has been used to obtain phosphomannan that is virtually free from glucan by interrupting the Pronase digestion after 4–5 h (in some cases a 2 h digestion was sufficient). Addition of fresh Pronase to the cell-wall residue then released a fraction that was rich in glucan, and this fraction was used for preliminary investigations on the glucan (T. N. Cawley, R. Letters & M. G. Harrington, unpublished work).

The phosphate group appears to have little effect on the precipitation of mannans from borate solutions by Cetavlon, as a 75% decrease in the number of phosphate groups in the mannan, by HF treatment, did not result in a decrease in the amount of mannan precipitated.

The serine and threonine contents of phosphomannan and of phosphomannan treated with alkali (Table 1) show that 70% of the serine and 78% of the threonine in the phosphomannan are lost by treatment with alkali. These results are explicable if it is

assumed that β -elimination of the alkoxide moiety of the phosphomannan occurred to give dehydro derivatives of serine and threonine. This type of elimination has been shown (Sentandreu & Northcote, 1968) to take place when a mannan-peptide fraction from yeast cell wall was treated with alkali; the formation of dehydro derivatives of the amino acids in the mannan-peptide during alkali treatment was shown by measuring the increase in E_{240} of the reaction mixture. Mannose, mannose disaccharides and possibly other mannose oligosaccharides were produced during the β -elimination reaction. Sentandreu & Northcote (1968) also demonstrated that reduction of the dehydro derivatives with potassium borohydride produced α -aminobutyric acid and an increase in the amount of alanine. These reactions have been used in the current investigation and have produced results quite similar to those reported by Sentandreu and Northcote (1968), thus indicating that the phosphomannan contains alkali-labile *O*-glycosyl bonds with serine and threonine. Comparison of the serine and threonine contents of isolated

Scheme 1. Reactions of phosphate monoesters of mannose and methyl mannoside

Reagents: (1) dicyclohexylcarbodi-imide in aq. pyridine; (2) dicyclohexylcarbodi-imide in aq. pyridine+triethylamine; (3) 0.1 M-HCl at room temp., 15 min.

cell wall and phosphomannan (Table 1) indicates that these alkali-labile bonds remain intact during the isolation of the phosphomannan, as there are only minor differences in the serine and threonine contents relative to the contents of the other amino acids in going from the cell wall to the phosphomannan fraction.

Phosphomannan preparations from yeast cell walls have been found to vary in molecular size. Thus the purified phosphomannan isolated from ethylenediamine extracts of cell walls of a strain of *Sacc. cerevisiae* was found to have a number-average molecular weight ($\bar{M}_n$) of 76000 by sedimentation and diffusion analysis (Sentandreu & Northcote, 1968). Hydrolysis of this phosphomannan in alkali decreased the molecular weight ($\bar{M}_n$) to 59000. The mannan released from walls of a strain of *Saccharomyces carlsbergensis* by digestion with a phosphomannanase was found to have a molecular weight greater than 200000 by gel filtration (McLellan & Lampen, 1968). Eddy & Longton (1969) used sedimentation and diffusion analysis to determine the molecular weights of the mannans released from a strain of *Sacc. carlsbergensis* ($\bar{M}_n$ 330000) and a strain of *Sacc. cerevisiae* ($\bar{M}_n$ 189000). Hydrolysis in alkali decreased these values to 120000 and 85000 respectively.

Gel filtration of the phosphomannan used in the present investigation indicated a molecular weight ($\bar{M}_n$) in excess of 200000 (Fig. 2). Hydrolysis in acid and in alkali decreased the size of the phosphomannan to a molecular weight of approx. 200000.

The phosphomannan was degraded to yield a reducing phosphate (I). The location of the phosphate group follows from investigations on its reaction with dicyclohexylcarbodi-imide in aq. pyridine, as outlined in Scheme 1. The formation of *N*-phosphorylureas on treatment with dicyclohexylcarbodi-imide is a general reaction of five-membered cyclic phosphate esters (Khorana *et al.*, 1957), such as compound (VI), which does not occur with six-membered cyclic phosphate esters such as compounds (IV) and (V). These observations establish that the reducing phosphate (I) produced by acid hydrolysis of phosphomannan has the phosphate group located on the primary hydroxyl group of the mannose moiety.

Results presented for the degradation of the phosphomannan by acid, alkaline and HF degradation and periodate oxidation are consistent with the structure (IX) (Scheme 2). This is a modification of the structure proposed by McLellan & Lampen (1968) and would give a periodate-oxidation product (X) in which the phosphate ester bond to the primary hydroxyl group would undergo β -aldehyde elimination by alkali and the hemiacetal-phosphate linkage would be labile under the acidic conditions used for determination of P_i . Our observations on the consumption of periodate by the phosphomannan and

on the formic acid produced are similar to those reported for the extracellular phosphomannan from *H. holstii* (Jeanes & Watson, 1962), and indicate that phosphodiester groups are linked between C-6 of one mannose unit and C-1 of another. An important difference between the structure proposed for the *H. holstii* phosphomannan and structure (IX) is the absence of a glycosidically linked mannose unit at C-2 of the terminal mannose residue. This difference is important in explaining the mechanism of hydrolysis of the phosphodiester bonds in alkali. Structure (IX) would account for these observations. The linkage between the terminal mannose unit and the phosphate group would be readily hydrolysed in alkali. In addition, the hydroxyl group on C-2 of the terminal mannose unit has a *trans* relationship with the phosphate group on C-1. In alkali this could result in displacement of the phosphate group from C-1 by rearside attack by the adjacent hydroxyl group, presumably with formation of 1,2-anhydro- β -D-mannopyranose (XI). Evidence for a reaction mechanism of this type has been obtained (Cawley & Letters, 1971) with a methyl α -D-mannopyranoside 6-(α -D-mannopyranosyl-1-phosphate) (VIII), a model compound of structure (IX). A similar mechanism has been suggested for the degradation of phenyl α -D-mannopyranoside in alkali (Ballou, 1954). The molecular size of the phosphomannan is decreased by hydrolysis in alkali and by autohydrolysis without removal of phosphate groups, as indicated by the retention volumes of the products on agarose-gel columns and by phosphorus contents. As much as one-third of the carbohydrate in the phosphomannan preparation was not recovered from hydrolysates when these products were isolated by precipitation with Cetavlon in the presence of borate. There are two possible explanations of these results. One is that a small mannan unit is glycosidically linked to the phosphate group. Disruption of this linkage, by alkali or by acid, would then result in a loss of carbohydrate without a loss of phosphate groups. The phosphate groups would be converted from the diester into the monoester form in the process. Alternatively, there may be a large mannan unit which has mannose oligosaccharide units attached to it by linkages that are labile in acid and in alkali. The conversion of the diester phosphate into monoester phosphate would then be a separate process from the removal of the smaller mannan unit (IX, Scheme 2).

The first possibility is unlikely, since autohydrolysis released approx. 1 mol of mannose/mol of diester phosphate hydrolysed. Similarly, when the phosphomannan was treated with 40% HF, which removed 70% of the phosphate groups, mannose (0.75 mol/mol of phosphate removed) was released, but no mannose oligosaccharides were detected. This dephosphorylation with 40% HF did not appreciably

Scheme 2. Degradation scheme for phosphomannan (IX) and related model compound (VIII). M = large mannose polysaccharide unit; m = mannose oligosaccharide units.

decrease the molecular size of the phosphomannan. Structure (IX), in which the phosphate groups are located on the outer region of the molecule, would fit these results. This structure (IX) is similar to that reported by Thieme & Ballou (1971) for *Kloeckera brevis* phosphomannan, which contains α -D-mannosyl 1-phosphate groups attached to C-6 of mannose units in a side chain which is in turn linked to mannose units in the 1 $\rightarrow$ 6-linked backbone.

The functional implications of a phosphomannan with structure (IX) are of interest in relation to flocculation of brewer's yeast. This phenomenon of yeast cells clumping together is dependent on the ultrastructure of the yeast cell wall. Lyons & Hough (1970) suggested a mechanism for flocculation involving a cross-bridging, via Ca^{2+} , between phosphomannan-proteins in the walls of adjacent yeast cells. This mechanism was based on their observations that higher concentrations of phosphorus were present in a surface fraction from cell walls of flocculent yeast and that walls from the flocculent yeast had a greater capacity to bind Ca^{2+} . Further investigations by Lyons & Hough (1971) showed that the capacity of cells to flocculate by cross-bridging was proportional to the number of phosphate groupings present in the outer mannan-protein layer of the cell wall. The concentration of phosphate in the outer layer was sufficient only in flocculent strains to enable binding of bivalent cations between adjacent cells. Structure (IX), in which the phosphate groups are located on the outside of the mannan molecule, would fit in with these observations. In contrast to the ease with which cell-wall phosphomannans bind Ca^{2+} it is surprising that when they are converted into the monoester form they are not degraded by alkaline phosphomonoesterase. This was first pointed out by McLellan & Lampen (1968) and has been confirmed in the present work. It would appear that the polysaccharide structure in the region of the phosphate groups prevents attack by alkaline phosphatase but does not interfere with binding of Ca^{2+} .

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