THE CELL WALL POLYSACCHARIDES OF CANDIDA ALBICANS: GLUCAN, MANNAN, AND CHITIN[1]

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ABSTRACT

Cells of *Candida albicans*, a pathogenic yeast, have been shown to contain, in addition to chitin, a glucan ($[\alpha]_D$ $-30°$) and a mannan ($[\alpha]_D$ $+78°$) in the approximate ratio of 1.00:0.64. The two polysaccharides were easily distinguishable by moving boundary electrophoresis in borate buffer and were separated from each other by fractionation of their copper complexes. Methylation and hydrolysis of the glucan yielded the following *O*-methyl ethers of D-glucose: 2,3,4,6-tetra-*O*-methyl (7 moles); 2,3,4-tri-*O*-methyl (13 moles); 2,4,6-tri-*O*-methyl (trace); 2,4-di-*O*-methyl (6 moles); and 2-*O*-methyl (1 mole). It was concluded that the glucan was a highly branched polysaccharide containing $\beta 1 \rightarrow 6$ and $\beta 1 \rightarrow 3$ linked residues. Periodate oxidation of the glucan supported this conclusion.

Methylation and hydrolysis of the mannan yielded the following *O*-methyl ethers of D-mannose: 2,3,4,6-tetra-*O*-methyl (1.65 moles); 3,4,6-tri-*O*-methyl (1.00 mole); 2,3,6-tri-*O*-methyl (0.18 mole); 3,4-di-*O*-methyl (1.90 moles). The mannan was therefore a highly branched polysaccharide with short chains of $\alpha 1 \rightarrow 2$ linked mannose residues joined together by $\alpha 1 \rightarrow 6$ linkages. Results of periodate oxidation agreed with this structure.

The differences between these two polysaccharides and glucans and mannans found in other yeasts are discussed.

Structural investigations of polysaccharide components of yeasts have been limited, with only one exception, to those in bakers' yeast (*Saccharomyces cerevisiae*) and there are a number of reports dealing with the glucan (1–4) and mannan (5–7) that occur in this species. The only report dealing with structures of polysaccharides from other yeast species appears to be that by Gorin and Perlin (8) which described a mannan produced by *Saccharomyces rouxii*. There have been other publications on the composition of cell walls of yeasts (9–18) and some of these (13–18) have dealt with species other than *Saccharomyces cerevisiae*. However, these papers were concerned primarily with examination of fractions of yeast cell walls by X-ray diffraction, electrophoresis, and chromatography of hydrolyzates; structures of polysaccharides were not investigated and constituent sugars were identified only by paper chromatography. The identification of D-arabinose as a constituent of polysaccharides found in *Nocardia asteroides* (19) and *Mycobacterium tuberculosis* (20) has shown that sugars can occur in unusual configurations in microorganisms. For this reason the distinction of enantiomorphic forms of naturally occurring sugars is of considerable significance and such distinction cannot be made by paper chromatography. It was therefore of interest to examine the polysaccharides of another species of yeast, *C. albicans*, to see if they differed in structure or constituent sugars from the polysaccharides of bakers' yeast. In addition to this a report that the polysaccharides of *C. albicans*, a pathogenic yeast, are serologically active (21) indicated that elucidation of their structures could be of immunological significance.

Isolation of Glucan and Mannan from C. albicans

Crude polysaccharides were isolated from powdered cells of *C. albicans* by the procedure given in the Experimental section. The product contained no nitrogen and hence was free from any proteinaceous material. Examination of this crude preparation by moving
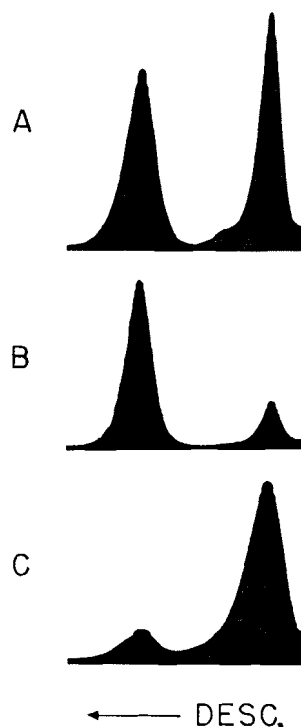
FIG. 1. Moving boundary electrophoresis of (A) crude polysaccharides, (B) mannan + trace of glucan, (C) glucan + trace of mannan.

boundary electrophoresis in borate buffer gave the separation pattern shown in Fig. 1A. The result clearly indicated two components; these were present in an approximate ratio of 0.61–0.67:1.00. Hydrolysis and chromatography of the crude polysaccharide preparation revealed the presence of mannose and glucose in a molar ratio of 0.50:1.00. The reasonable agreement between this ratio and the ratio of the two components found by electrophoresis indicated that these two components were a glucan and a mannan. This conclusion was confirmed by the isolation of a glucan and a mannan by fractionation with Fehling's solution. Fractions in which each polysaccharide still contained traces of the other were used to identify the two peaks in the electrophoretic separation. Figure 1B is the electrophoretic pattern given by the mannan-rich fraction and Fig. 1C is that given by the glucan-rich fraction. All three separations shown in Fig. 1 were photographed after the same time interval; the results show clearly that the component of greater mobility was the mannan, and the one with lower mobility the glucan. Electrophoretic mobilities of the two components were the same after fractionation as before, an indication that the separation procedure had not altered the polysaccharides.

Isolation of a glucan and a mannan from *C. albicans* has not been reported before. Jonsen *et al.* (15) isolated a polysaccharide preparation from the same organism and identified glucose and mannose by paper chromatography after hydrolysis. However, these authors obtained a single peak on electrophoresis of the polysaccharide in acetate buffer and accepted this as indicative of homogeneity. Polysaccharides, unless they contain acidic groups, would be expected to have very similar mass:charge ratios and hence would not be separable by electrophoresis in acetate buffer. The electrophoretic

separations reported in the present communication depended on the polysaccharides being complexed to different extents with borate (22) thereby acquiring different mass: charge ratios which permitted their separation. More recently Kessler and Nickerson (18) have reported the isolation of glucomannan–protein and glucan–protein complexes from three strains of *C. albicans*. However, the fractionation procedure used, alkali extraction and ammonium sulphate precipitation, was not designed to separate the polysaccharides nor was any criterion of homogeneity of the fractions reported. It is very likely, in view of the present work, that the glucomannans reported (15, 18) in *C. albicans* were mixtures of a glucan and mannan.

The glucan and mannan found in *Saccharomyces cerevisiae* have been established as components of the cell wall (9); this is also thought to be true for the glucan and mannan from *C. albicans* because an isolated cell wall preparation yielded glucose and mannose on hydrolysis.

A small amount of chitin was isolated from the cell wall of *C. albicans* and identified by X-ray diffraction. These results agree with those of other workers (12, 13, 14) who have reported the presence of chitin in small and variable amounts in the cell walls of a number of yeast species, including *C. albicans*.

Glucan from C. albicans

The glucan gave a number average degree of polymerization of 30 ± 2. On acid hydrolysis it yielded D-glucose, characterized as the *p*-nitroanilide and by specific rotation, as the only detectable sugar. Its negative rotation ($[\alpha]_D$ $-30°$) indicated that a high proportion of β-glycosidic bonds were present and its stability to hot dilute oxalic acid showed that furanose ring forms were absent.

When oxidized by periodate the glucan consumed 1.60 moles of oxidant and yielded 0.72 mole of formic acid per mole anhydroglucose. The latter value indicated that about 70% of the D-glucose units were non-reducing terminal units or were joined to adjacent units by $1 \rightarrow 6$ linkages. The very small amount of oxidant not accounted for by formic acid production indicated that most, if not all, of the remaining 28% of glucose units were substituted so that they were resistant to oxidation by periodate. This conclusion was confirmed by reduction and hydrolysis of the periodate-oxidized polysaccharide (23) which yielded glycerol and glucose as the only products detectable by paper chromatography. The glycerol arose from C_4, C_5, and C_6 of those glucose units which were terminal or 1,6-disubstituted and the glucose represented those units in the glucan that were not oxidized. No erythritol could be found in the hydrolyzate of the oxidized, reduced polysaccharide and therefore no $1 \rightarrow 4$ linkages were present in the glucan (23). The above results showed that in the glucan 72% of the glucose units were non-reducing terminal units or were joined by $1 \rightarrow 6$ linkages, the remainder being $1 \rightarrow 3$ linked or highly substituted in a branched structure.

The glucan was methylated and hydrolyzed to give the methyl ethers listed in Table I.

TABLE I

Hydrolysis products of methylated glucan

2,3,4,6-Tetra-*O*-methyl-D-glucose	(7 moles)
2,3,4-Tri-*O*-methyl-D-glucose	(13 moles)
2,4,6-Tri-*O*-methyl-D-glucose	(Trace)
2,4-Di-*O*-methyl-D-glucose	(6 moles)
2-*O*-Methyl-D-glucose	(1 mole)

The main products shown here must have arisen from the following structural units in the polysaccharide:

$$\text{Glp1--} \quad 7 \qquad\qquad \overset{6}{\text{Glp1--}} \quad 13 \qquad\qquad \overset{6}{\underset{3}{\text{Glp1---}}} \quad 6$$

I   II   III
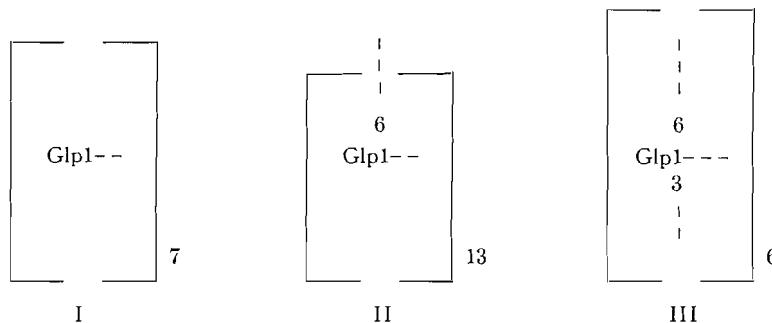
Thus 2,3,4,6-tetra-O-methyl-D-glucose represented non-reducing terminal units substituted only in position 1 (I); 2,3,4-tri-O-methyl-D-glucose came from units that were joined through positions 1 and 6 (II); 2,4-di-O-methyl-D-glucose arose from units that were linked through positions 1, 3, and 6 (III). The significance of the 2,4,6-tri-O-methyl-D-glucose and the 2-O-methyl-D-glucose is difficult to assess because of the small amounts found and because the methylated glucan, methylated to constant methoxyl and showing no hydroxyl absorption in the infrared, still contained only 40.5% methoxyl (calc. 45.6%). It is possible that the polysaccharide contained an impurity other than protein (e.g. lipid) which caused this low methoxyl. A glucan, containing the above three structural units in the amounts indicated, would consume 1.54 moles of periodate per mole anhydroglucose with production of 0.77 mole of formic acid per mole anhydroglucose. These are in good agreement with the values of 1.60 and 0.72 that were found for periodate consumption and formic acid production respectively in the purified glucan. If there was an impurity in the glucan which caused the low methoxyl then it must have consumed periodate, otherwise these values would be lower than theoretical. The results of the methylation study therefore confirmed the conclusions based on periodate oxidation and showed clearly that the glucan from *C. albicans* was a highly branched polysaccharide formed from $\beta\ 1 \rightarrow 6$ and $\beta\ 1 \rightarrow 3$ linked glucose residues. This structure is quite different from those proposed by various workers (1, 2, 3, 4) for the glucan from *Saccharomyces cerevisiae*. Hassid *et al.* (1) found 2,4,6-tri-O-methyl-D-glucose as the only product from methylation and hydrolysis of that glucan and therefore proposed a straight chain, $\beta\ 1 \rightarrow 3$ linked structure. On the other hand, Bell and Northcote (2) reported similar experiments in which the products from methylation and hydrolysis showed that the glucan was branched with chains of $\beta\ 1 \rightarrow 3$ linked units being joined by $1 \rightarrow 2$ interchain links. Peat *et al.* (3) have shown by fragmentation analysis that the glucan from *Saccharomyces cerevisiae* was a linear polysaccharide formed by $\beta\ 1 \rightarrow 3$ and $\beta\ 1 \rightarrow 6$ glycosidic linkages. A succeeding report (4) showed that some 10–20% of the linkages were $\beta\ 1 \rightarrow 6$. The glucan from *C. albicans* differs from these three structures reported for the glucan from *Saccharomyces cerevisiae* in being much more highly branched and having a preponderance (73%) of $\beta\ 1 \rightarrow 6$ linkages.

Mannan from C. albicans

The mannan showed a number average degree of polymerization of 41 ± 2 and had a

specific rotation of $+78° \pm 2°$. Mannose was the only sugar detectable by paper chromatography and electrophoresis after acid hydrolysis and it was proved to be D-mannose by the specific rotation ($+13° \pm 2°$) and by isolation of its crystalline phenylhydrazone. The decrease in rotation after hydrolysis indicated that α-glycosidic bonds were predominant. The mannan was stable to hot, dilute oxalic acid showing that no furanose ring forms were present.

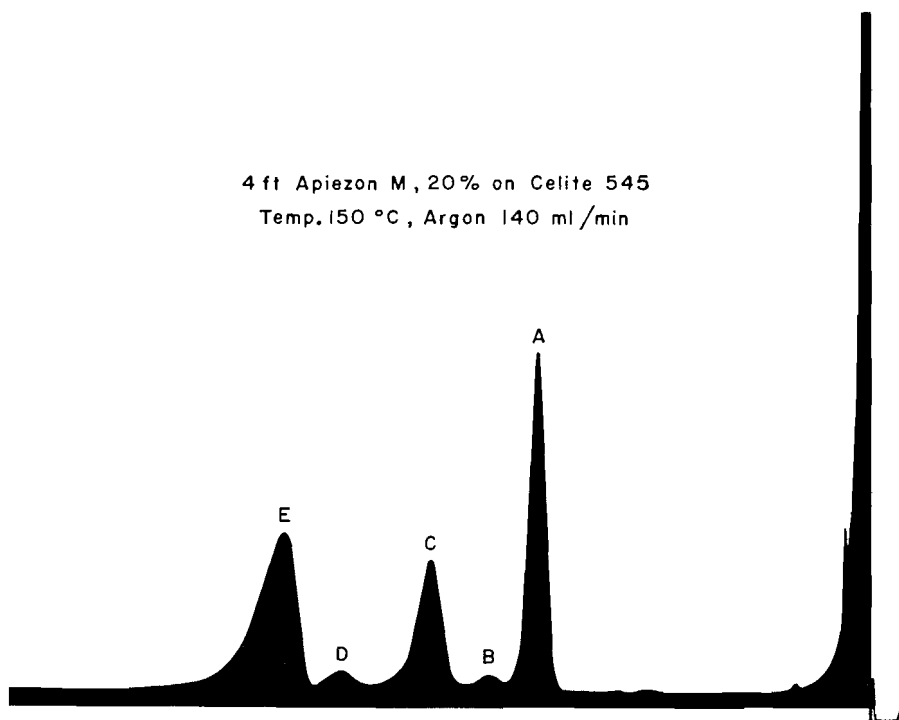On methylation and hydrolysis the mannan yielded the components shown in Fig. 2.

4 ft Apiezon M , 20% on Celite 545
Temp. 150 °C , Argon 140 ml /min

FIG. 2. Separation of methanolysis products from methylated mannan by gas–liquid partition chromatography.

		Molar ratio
(A)	Methyl-2,3,4,6-tetra-*O*-methyl-α-D-mannopyranoside	1.65
(B)	Unknown	0.13
(C)	Methyl-3,4,6-tri-*O*-methyl-α-D-mannopyranoside	1.00
(D)	Methyl-2,3,6-tri-*O*-methyl-α-D-mannopyranoside	0.18
(E)	Methyl-3,4-di-*O*-methyl-α-D-mannopyranoside	1.90

This is a reproduction of the separation curve from gas–liquid partition chromatography of the methanolysis products from the methylated mannan. This technique was developed only recently (24, 25) and was not available for use when the glucan from *C. albicans* was being investigated. Components A, C, and E were identified by isolation of crystalline derivatives and represented the main structural units of the mannan. The quantities of components B and D were too small to permit unequivocal identifications but component D was identical on gas–liquid partition chromatograms with an authentic sample of methyl-2,3,6-tri-*O*-methyl-α-D-mannopyranoside. The main structural features of the mannan could therefore be represented by the following units.

Man p-1－－－－　　　15

I

Man p-1－－－－
|
2
|
|
|
9

II

|
|
|
6
|
Man p-1－－－
|
2
|
|
|
17

III
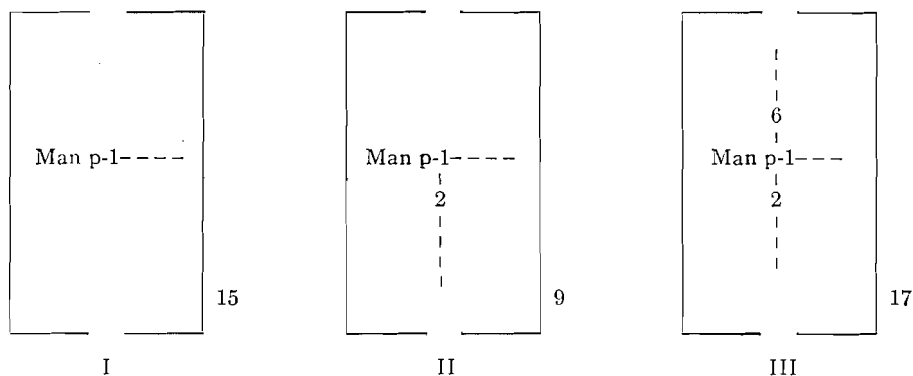
Thus components A, C, and E in Fig. 2 represented structural units I, II, and III respectively. The significance of components B and D in Fig. 4 is uncertain because of the possibility that they were products of incomplete methylation or of demethylation on hydrolysis. The amount of non-reducing terminal end groups (I) is slightly less than required to account for the branch points (III). A loss of 13% of the 2,3,4,6-tetra-O-methyl-D-mannose during evaporations of the formic acid hydrolyzate could account for this. It is possible that such discrepancies have not been detected previously because of the lack of accurate quantitative data as supplied by gas–liquid partition chromatography. A polysaccharide composed of structural units I, II, and III should consume 1.37 moles of periodate per mole anhydromannose with production of 0.37 mole of formic acid per mole anhydromannose. The values actually found, 1.13 and 0.27 moles per mole anhydromannose, respectively, were slightly lower and could have been caused by an impurity present in the mannan during periodate oxidation but removed during methylation, or again could be a result of greater accuracy of the gas–liquid partition chromatography data as compared with the periodate oxidation data. After periodate oxidation the resulting polyaldehyde was reduced and hydrolyzed (23). Glycerol was the only polyol detectable in the hydrolyzate, in agreement with the finding from methylation that only $1 \to 2$ and $1 \to 6$ bonds were present. It was clear from the foregoing data that the mannan possessed a highly branched structure in which relatively short chains of $\alpha\ 1 \to 2$ linked mannose units were joined together by $\alpha\ 1 \to 6$ linkages. The mannan from *C. albicans* is therefore somewhat different from the mannan isolated from bakers' yeast (*Saccharomyces cerevisiae*) (5, 6, 7) and more closely resembles that found in *Saccharomyces rouxii* (8). All of these mannans exhibited high positive specific rotations, which indicated the presence of α-glycosidic linkages, and this has been confirmed unequivocally (8) for the $1 \to 2$ linkage in the mannan from *Saccharomyces rouxii*. The difference lies in the tri-O-methyl-D-mannoses found after hydrolysis of the methylated mannans. The methylated mannans from bakers' yeast (5, 6, 7) yielded equimolar amounts of 2,4,6- and 3,4,6-tri-O-methyl-D-mannose indicative of an equal number of $1 \to 3$ and $1 \to 2$ linkages in the unbranched portion of the polysaccharide. On the other hand, the methylated mannan from *Saccharomyces rouxii* (8) yielded 2,4,6- and 3,4,6-tri-O-methyl-D-mannose in a ratio of 2:7 indicating that $1 \to 2$ linkages predominated over $1 \to 3$. The mannan from *C. albicans* yielded no 2,4,6-tri-O-methyl-D-mannose after methylation and hydrolysis and therefore could not have contained any $1 \to 3$ linkages. This conclusion was substantiated by the absence of mannose in the hydrolyzate of the periodate-oxidized mannan because any $1 \to 3$ linked mannose residues would have survived the oxidation. This

absence of $1 \rightarrow 3$ linkages and the high degree of branching constitute the main differences between the mannan from *C. albicans* and those so far examined from other yeasts.

EXPERIMENTAL

Paper chromatograms were run by the descending method using the following solvent systems (v/v ratio):

(A)   butan-1-ol:pyridine:water, 6:4:3;
(B)   butan-1-ol:ethanol:water, 9:3:3;
(C)   2-butanone:water, azeotrope.

Paper electrophoreses were done on Whatman 3 MM paper in 0.1 *M* borate buffer (22) using a potential gradient of 25 v/cm for 1 hour. Sugars were detected on chromatograms and electrophorograms by the *p*-anisidine hydrochloride spray reagent (26). Non-reducing compounds were detected on chromatograms by silver nitrate : sodium hydroxide sprays (27). Moving boundary electrophoreses were carried out in 0.05 *M* borate buffer in a Tiselius-type, Spinco Model H apparatus. Evaporations were carried out under diminished pressure at 35° C or less on a rotary film evaporator. Melting points are corrected and specific rotations are equilibrium values unless stated otherwise.

Isolation of Crude Polysaccharides

C. albicans was cultured in a liquid medium containing the following nutrients: 4% cerelose (crude glucose), 2% neopeptone "Difco", 0.05% yeast extract, 0.001% thiamin, 0.005% inositol. After incubation at 37° C for 5–12 days the cultures were autoclaved at 121° C for 20 minutes; cells were then separated in a continuous centrifuge, freeze-dried, and ground in a ball mill for 16 hours. Extraction with petroleum ether (30–60° C) removed lipid, and digestion with trypsin destroyed protein. The residue from these two treatments was then extracted with boiling 3% aqueous sodium hydroxide to solubilize the polysaccharides, which were freed from low molecular weight impurities by dialysis and precipitation with ethanol. The polysaccharide preparation thus obtained (6.3% of the dried, powdered cells) was a light tan powder which contained no nitrogen as shown by microanalysis. A sample of this material was hydrolyzed by *N* hydrochloric acid at 97° C in a sealed tube for 16 hours. Paper chromatography (solvent A) and paper electrophoresis of the hydrolyzate showed two components which were identical with samples of glucose and mannose run on the same paper strips. Another sample (150 mg) of the polysaccharide preparation was dissolved in 0.05 *M* sodium tetraborate, equilibrated with the buffer by dialysis, and examined by moving boundary electrophoresis. Figure 1A shows the complete separation of two components that was obtained by this procedure. The electrophoretic mobilities (μ) of these two components were respectively 4.38 and 9.34×10^{-5} cm^2 v^{-1} sec^{-1}. Areas under the two peaks were measured from diagrams obtained at two different times and gave ratios of the two components of 0.61:1.00 and 0.67:1.00. The molar ratio of mannose to glucose in the hydrolyzate of the polysaccharide mixture was 0.50:1.00 as determined by the densitometer method of Martin (28).

Separation of Glucan and Mannan

The crude polysaccharides (8.0 g) were stirred vigorously in water (1200 ml) for 3 hours. Insoluble material was removed by centrifugation and dried by solvent exchange with ethanol and ether (yield, 0.9 g). Paper electrophoresis of a hydrolyzate of a sample from this fraction showed that glucose was the only component sugar present. Hydrolysis and paper electrophoresis of the material in solution showed the presence of both glucose and mannose. An attempt was made to separate the mixture of polysaccharides in aqueous

solution by fractional precipitation with ethanol (29) but the fractions obtained showed no variation in glucose and mannose composition. These fractions were therefore combined, redissolved in water, and the polysaccharides were precipitated as copper complexes by addition of Fehling's solution. This method was used successfully by Haworth *et al.* (5) to isolate a mannan from bakers' yeast. The precipitated copper complex was washed repeatedly with water and at the sixth washing the precipitate dissolved. This solution was poured into 5 volumes of 10% methanolic hydrogen chloride to precipitate the polysaccharide which was dried by solvent exchange with ethanol and ether (yield, 2.13 g). Paper electrophoresis of a hydrolyzate of a sample from this fraction showed that mannose was the only component sugar present. The washings from the precipitated copper complex were combined and the polysaccharides were recovered by precipitation with acidified ethanol. This material was then recomplexed with copper and washed with water in the same way as before. Four repetitions of this procedure yielded two more polysaccharide fractions, one yielding mainly mannose but with a trace of glucose on hydrolysis and the other yielding mainly glucose with a trace of mannose. To preserve the chemically pure glucan and mannan for structural studies these impure fractions were used to determine the identities of the two peaks found in electrophoresis. The electrophoretic diagram shown in Fig. 1B is that given by the fraction yielding mainly mannose on hydrolysis but with a trace of glucose. Figure 1C shows the electrophoretic pattern given by the fraction yielding glucose on hydrolysis with only a trace of mannose. Fractionation in the same way of another 6.0 g of crude polysaccharides provided further quantities of pure glucan (0.60 g) and mannan (1.5 g).

Properties of Purified Glucan and Mannan

Glucan

This polysaccharide had $[\alpha]_D^{26} = -30°\pm2°$ (c, 1.0% in N sodium hydroxide) changing to $[\alpha]_D^{26} = +53°\pm2°$ (c, 2.0% in N hydrochloric acid) after hydrolysis in N hydrochloric acid at 97° C for 16 hours. The hydrolyzate was neutralized (Amberlite IR-45 ion exchange resin), evaporated to dryness, and the residue was heated with p-nitroaniline in methanol solution for 40 minutes. The solution was cooled and the crystals which separated were recrystallized from methanol to yield N-p-nitrophenyl-D-glucopyranosylamine dihydrate (30), m.p. 183–184° C, $[\alpha]_D^{25} = -199°\pm1°$ (c, 1.0% in pyridine). Attempted partial hydrolysis of the glucan by 0.025 N oxalic acid at 97° C for 3 hours released no cleavage products detectable by paper chromatography. The degree of polymerization of the glucan was 30 ± 2 as determined by hypoiodite oxidation of the reducing end group in phosphate buffer (31).

Mannan

This polysaccharide had $[\alpha]_D^{26} = +78\pm2°$ (c, 1.1% in water) changing to $[\alpha]_D^{26} = +13°\pm2°$ (c, 2.0% in N hydrochloric acid) after hydrolysis in N hydrochloric acid at 97° C for 16 hours. The hydrolyzate was neutralized (Amberlite IR-45 ion exchange resin), mixed with an equal volume of an aqueous solution of phenylhydrazine acetate, and allowed to stand at 25° C for 20 hours. The crystals which formed were recrystallized twice from water:ethanol to yield mannose phenylhydrazone (32), m.p. 198–199° C (d), $[\alpha]_D^{24} = +25°\pm2°$ (c, 1.0% in pyridine). Like the glucan, the mannan showed no evidence of partial hydrolysis when heated with 0.025 N oxalic acid at 97° C for 3 hours. The degree of polymerization of the mannan, estimated in the same way as for the glucan, was 41 ± 2.

Periodate Oxidations

The glucan (111.2 mg, 0.66 mmole) and mannan (122.3 mg, 0.75 mmole) were dissolved separately in water (125 ml) and 0.25 M sodium periodate (25 ml) was added to each. Reagent blanks were also prepared and oxidations were carried out at 25° C in the absence of light. At the intervals noted below samples were removed for estimation of formic acid and periodate. For estimation of formic acid the excess periodate in a 10-ml aliquot was destroyed by 2,3-butanediol, a few crystals of potassium iodide were added and the liberated iodine was titrated to a starch end point with 0.01 N thiosulphate. For periodate estimations (33, 34) sodium bicarbonate (1.7 g), 0.1 N sodium arsenite (5 ml), and a few crystals of potassium iodide were added to 10-ml aliquots from the oxidations. The solutions were stored in the dark for 30 minutes and were then titrated with 0.022 N iodine solution to a starch end point. The results, given in moles per anhydrohexose unit were as follows:

Time, hours	Glucan		Mannan	
	Formic	Periodate	Formic	Periodate
24	0.68	1.60	0.21	1.10
44	0.71	1.58	0.26	1.10
70	0.72	1.61	0.27	1.13

After the oxidations were complete excess periodate was destroyed by 2,3-butanediol and salts and acetaldehyde were removed by dialysis. The oxidized polysaccharides were then reduced by potassium borohydride, hydrolyzed by N hydrochloric acid at 97° C for 3 hours and the hydrolyzates were examined by paper chromatography (solvent B, silver nitrate : sodium hydroxide sprays) (23). By this sequence of reactions the glucan yielded glycerol and glucose while the mannan gave only glycerol. Erythritol was not found in either hydrolyzate.

Methylation of Glucan and Mannan

Glucan

The glucan (893 mg) was methylated five times for 24 hours in 30% sodium hydroxide (20 ml) and dimethyl sulphate (10 ml). The product was isolated by extraction into chloroform and was treated for 24 hours in that solvent (10 ml) with methyl iodide (6 ml) and silver oxide (3.0 g). After three further methylations with methyl iodide and silver oxide, the methylated polysaccharide was precipitated as a white powder (1.0 g, methoxyl = 40.5%) from chloroform solution by addition of petroleum ether (30–60° C). The methoxyl content of this product was not increased by another methylation and an infrared spectrum showed only a trace of hydroxyl absorption. The powdery product was extracted with petroleum ether and then successively with petroleum ether containing increasing amounts of chloroform. All fractions showed identical hydrolysis products on paper chromatograms and the main portion of the product dissolved in petroleum ether : chloroform mixtures of 6:4, 5:5, and 4:6. These three fractions were combined and reprecipitated to give a white powder (660 mg, methoxyl 40.5%) showing no hydroxyl absorption in the infrared.

Mannan

The mannan (2.14 g) was methylated five times with 30% sodium hydroxide

(5×100 ml) and dimethyl sulphate (5×50 ml) in the same way as described for the glucan. The product (2.29 g) showed distinct hydroxyl absorption in the infrared so it was methylated again, this time in tetrahydrofuran (30 ml) with sodium hydroxide (10 g) and dimethyl sulphate (15.5 ml) (35). The product still showed hydroxyl absorption in the infrared and had methoxyl equal to 39.7% so it was methylated again in tetrahydrofuran as before. This methylation gave a product (2.20 g) having no hydroxyl absorption in the infrared and with methoxyl equal to 40.4%, unchanged by another methylation. The methylated polysaccharide was extracted with boiling petroleum ether (30–60° C). The extract was removed by decantation and evaporated to yield an oily product (methoxyl = 29%) which was discarded. The residue from this extraction was stirred in ether and the insoluble portion was removed by centrifugation. The ethereal supernatant was evaporated to yield the methylated mannan (1.56 g, methoxyl = 43.2%), which showed no hydroxyl absorption in the infrared.

Hydrolysis Products from Methylated Glucan

The methylated glucan (650 mg) was hydrolyzed by formic acid according to the procedure described by Jones and Wilkie (36). Qualitative and quantitative (37) paper chromatography gave approximate molar ratios of the methyl ethers of glucose shown in Table I.

The mixture of methylated sugars (645 mg) was resolved on a cellulose column (2.3×28 cm) using solvent C to give four fractions. *Fraction 1* (73.5 mg) contained only 2,3,4,6-tetra-*O*-methyl-D-glucose; *fraction 2* (126.4 mg) contained 2,3,4,6-tetra-*O*-methyl-D-glucose and 2,3,4-tri-*O*-methyl-D-glucose; *fraction 3* (236.8 mg) contained 2,3,4-tri-*O*-methyl-D-glucose and a trace of 2,4,6-tri-*O*-methyl-D-glucose; *fraction 4* (126.7 mg) contained 2,4-di-*O*-methyl-D-glucose with a trace of 2-*O*-methyl-D-glucose (recovery) = 563.4 mg, 87.4%). Those fractions which were mixtures were resolved further by preparative paper chromatography on Whatman 3 MM paper using solvent C, and individual components were identified as described below.

2,3,4,6-Tetra-O-methyl-D-glucose

The unknown showed the same chromatographic mobility in solvents A, B, and C as an authentic sample of 2,3,4,6-tetra-*O*-methyl-D-glucose. The product crystallized from petroleum ether (30–60° C) and was recrystallized from *N*-hexane to give a compound with a melting point of 88–89° C and $[\alpha]_D^{24} = +82°\pm2°$ (*c*, 0.16% in water). The melting point was unchanged by admixture with an authentic sample of 2,3,4,6-tetra-*O*-methyl-D-glucose.

2,3,4-Tri-O-methyl-D-glucose

The sirupy unknown had R_g values of 0.90 and 0.75 in solvents B and C respectively. Authentic samples of tri-*O*-methyl-D-glucoses showed the following R_g values in the same solvents: 2,3,4—0.90, 0.75; 2,4,6—0.85, 0.62; 2,3,6—0.88, 0.70. The unknown (97.3 mg) was refluxed for $1\frac{1}{2}$ hours with aniline (41.0 mg) in ethanol (2 ml). The solvent was evaporated and the crystalline residue was recrystallized from ether to give a product having a melting point of 144.5–145.5° C and $[\alpha]_D^{26} = +70°\pm1°$ (*c*, 0.83% in ethanol). Peat *et al.* (38) report a melting point of 145–146° C for *N*-phenyl-2,3,4-tri-*O*-methyl-D-glucosylamine. Mother liquors from the above crystallization yielded a product having a melting point of 137–138° C unchanged by repeated recrystallization. The analysis and infrared spectrum of this material were identical with those of the product melting at 144.5–145.5° C and it appeared to be another crystalline form of the same compound. Analysis: Calc. for $C_{15}H_{23}O_5N$: C, 60.59%; H, 7.74%. Found: C, 60.61%, H, 7.42%.

2,4,6-Tri-O-methyl-D-glucose

Only a trace of this compound was present. It gave the same color reaction with *p*-anisidine hydrochloride on paper chromatograms and had the same R_g values (0.85, 0.62) in solvents B and C as an authentic sample of 2,4,6-tri-*O*-methyl-D-glucose.

2,4-Di-O-methyl-D-glucose

The unknown sample gave the same color reaction with *p*-anisidine hydrochloride on paper chromatograms as 2,4-di-*O*-methyl-D-glucose. In solvents A, B, and C it had R_g values of 0.81, 0.67, and 0.31 respectively. Authentic samples of di-*O*-methyl-D-glucoses had the following R_g values in the same solvents: 2,4—0.81, 0.67, 0.31; 2,3—0.83, 0.72, 0.36; 3,6—0.79, 0.66, 0.28. The unknown product (96.6 mg) was dissolved in ethanol (3 ml) to which *p*-nitroaniline (80 mg) and glacial acetic acid (2 drops) were added. The solution was kept at 25° C for $1\frac{1}{2}$ hours, heated on a steam bath for 2 hours and stored at room temperature overnight. A brown precipitate was filtered and washed with ethanol and ethyl acetate which removed much of the color. It was found that the light tan residue, consisting of microcrystals, could be purified by sublimation at 0.01 mm pressure at 200° C (bath temperature) to give *N*-*p*-nitrophenyl-2,4-di-*O*-methyl-D-glucosylamine, m.p. 250–251° C (*d*) (39).

2-O-Methyl-D-glucose

The unknown sample had the same R_g value (0.55) in solvent A as 2-*O*-methyl-D-glucose and gave the same color reaction with *p*-anisidine. R_g values of mono-*O*-methyl glucoses in solvent A were as follows: 2-*O*-methyl-, 0.55; 3-*O*-methyl-, 0.57; 4-*O*-methyl-, 0.51; 6-*O*-methyl-, 0.49. Attempts to isolate the compound in crystalline form or to prepare a crystalline derivative were unsuccessful because of insufficient material.

Hydrolysis Products from Methylated Mannan

The methylated mannan (1.54 g) was hydrolyzed by the formic acid procedure of Jones and Wilkie (36). A portion (100 mg) of the hydrolyzate was refluxed with 4% methanolic hydrogen chloride for 16 hours. The resulting mixture of methyl glycosides was resolved by gas–liquid partition chromatography (24, 25), which gave the separation and quantitative data shown in Fig. 2. To identify the constituents of the mixture the remainder (1.44 g) of the hydrolyzate was fractionated on a cellulose column using solvent C to give five fractions:

Fraction 1, tetra-*O*-methyl-D-mannose (0.150 g).
Fraction 2, tetra- and tri-*O*-methyl-D-mannoses (0.692 g).
Fraction 3, tri-*O*-methyl-D-mannose (0.065 g).
Fraction 4, tri- and di-*O*-methyl-D-mannoses (0.241 g).
Fraction 5, di-*O*-methyl-D-mannose (0.207 g).
Recovery = 1.355 (94.1%).

The major components in these fractions were identified as follows:

2,3,4,6-Tetra-O-methyl-D-mannose

On paper chromatograms fraction 1 showed only one component having R_g values of 0.99 and 0.98 in solvents B and C respectively. The specific rotation was $[\alpha]_D^{28} = +30° \pm 1°$ (*c*, 3.0% in methanol) and the product yielded a crystalline anilide, m.p. 144–145° C, $[\alpha]_D^{26} = +42° \rightarrow 8°$ (*c*, 0.72% in methanol). These values are in agreement with those reported for 2,3,4,6-tetra-*O*-methyl-D-mannose (40) and its anilide (41). After methanolysis fraction 1 showed one peak, corresponding to peak A, Fig. 2, on gas–liquid partition chromatography.

3,4,6-Tri-O-methyl-D-mannose

On paper chromatograms fraction 3 was identical with 3,4,6-tri-*O*-methyl-D-mannose and had R_g values of 0.90 and 0.76 in solvents B and C respectively. The product crystallized when nucleated with an authentic sample of 3,4,6-tri-*O*-methyl-D-mannose. Recrystallization from ether:hexane yielded a product with a melting point of 102–104° C and $[\alpha]_D^{28} = +33° \pm 3°$ (*c*, 3.2% in methanol), which are in agreement with the reported values (42). This compound showed only one peak, corresponding to peak C, Fig. 2, on gas–liquid partition chromatography after methanolysis.

3,4-Di-O-methyl-D-mannose

R_g values of fraction 5 on paper chromatograms were 0.74 and 0.44 in solvents B and C respectively, identical with those given by authentic 3,4-di-*O*-methyl-D-mannose. The product crystallized when nucleated with an authentic sample and recrystallization from ethyl acetate yielded a product with a melting point of 70–73° C (8, 43), and $[\alpha]_D^{28} = +32 \pm 1°$ (*c*, 2.0% in methanol) (6). This compound was methanolyzed and the methyl glycoside gave one peak, corresponding to peak E, Fig. 2, on gas–liquid partition chromatography.

Isolation of Chitin from C. albicans

Chitin was isolated from dried *C. albicans* cells (9.0 g) by the procedure of Scholl (44), which involved exhaustive extraction with boiling 10% potassium hydroxide, oxidation with 1% aqueous potassium permanganate, and removal of manganese dioxide by dilute (0.8%) hydrochloric acid. The residue from these reactions was dried with ethanol and ether to yield a product (545 mg, 6.05%) which was shown by X-ray diffraction to contain chitin and traces of corundum.

Isolation and Hydrolysis of Cell Walls from C. albicans

Cells of *C. albicans* were extracted with petroleum ether to remove lipid, incubated with trypsin to destroy protein, and suspended in water at a concentration of 1%. This suspension was shaken with fine glass beads for 45 minutes in a Mickle disintegrator (45). The glass beads were then removed by filtration on sintered glass and the turbid filtrate was centrifuged for 1 minute at 1000 r.p.m. Microscopic examination of the precipitate revealed the presence of many whole cells but further centrifugation of 1 minute at 1000 r.p.m. yielded a precipitate in which no intact cells could be detected. Hydrolysis and paper chromatography of this precipitate revealed the presence of glucose and mannose. The supernatant liquid from the second precipitate was centrifuged for 15 minutes at 15,000 r.p.m. to yield a creamy precipitate which yielded neither glucose nor mannose after hydrolysis.

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