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THE STRUCTURE OF THE CAPSULAR POLYSACCHARIDE FROM *Cryptococcus neoformans* SEROTYPE D

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ABSTRACT

The capsular polysaccharide from *Cryptococcus neoformans* serotype D has been studied by employing the usual methods for the elucidation of chemical structure. The results are consistent with the occurrence of a polysaccharide having both D-glucosyluronic acid and D-xylosyl groups present as nonreducing end-groups attached to O-2 of D-mannosyl residues which are linked α-D-(1 → 3) in a linear backbone.

INTRODUCTION

In two earlier reports[1,2], we described the structure of the capsular polysaccharide from *Cryptococcus neoformans* serotype C. Four serotypes (A, B, C, and D) of *C. neoformans* have been described[3,4], and two different, perfect states, namely, *Filobasidiella neoformans* and *F. bacillispora* have recently been discovered among the four serotypes[5,6]. Isolates of serotypes A and D produce the same perfect state *F. neoformans*, and also share the same natural reservoir, which is not shared[7] by isolates of serotypes B and C. The latter serotypes were found to produce a perfect state called *F. bacillispora*. On the basis of these and other biochemical differences, *C. neoformans* serotypes have now been divided into two groups[8]; serotypes A and D belong to *Cryptococcus neoformans*, whereas serotypes B and C belong to *Cryptococcus bacillisporus*.

The chemical structures of all of the capsular polysaccharide antigens from these four serotypes have not yet been worked out. In recent papers[1,2], we described the structure of the capsular polysaccharide serotype C. Merrifield *et al.*[9] reported on the structure of a capsular polysaccharide from *C. neoformans*, determined here to be serotype A; they showed that it is composed of mannose, xylose, and glucuronic acid residues in the ratios 3:2:1, and that it has an α-(1 → 3)-linked mannan backbone substituted at O-2 of *each* residue by either glucuronic acid or xylose. Blandamer and Danishefsky[10] reported on the partial structure of the capsular polysaccharide from *C. neoformans* serotype B. On the basis of the composition of mono- and oligo-saccharides, it was suggested that this polysaccharide also contains a mannan backbone having

branches of xylose and glucuronic acid. In the present paper, we describe our findings on the structure of the capsular polysaccharide from *C. neoformans* serotype D.

EXPERIMENTAL

General methods. — All evaporations were conducted in a rotary evaporator at or below 40° (bath temperature). Optical rotations were measured with a Perkin–Elmer 141 polarimeter at 20 ± 1° using sodium light (589 nm). ^{1}H.N.m.r. spectra were recorded with a Jeol JNM FX-100 spectrometer, using a 2% solution of the polysaccharide in D_2O at[11] 95°; the polysaccharide hydrogen was "exchanged" three times from D_2O. The τ values were measured downfield from the signal given by tetramethylsilane ($\tau = 10$).

Carboxyl-reduced polysaccharide was prepared by the method of Taylor and Conrad[12], three treatments with the reagents being needed for complete reduction.

Immunoelectrophoresis was performed with 1% agarose in 0.1M Tris acetate buffer, pH 8.3. The same buffer was used for electrophoresis at a constant 6 V.cm^{-1} for 1.5 h. Rabbit antiserum to whole cells of *C. neoformans* serotype D (kindly donated by Dr. J. E. Bennett) was used, and precipitation was allowed to proceed for 48 h at 5°.

Polysaccharide isolation. — The polysaccharide was obtained from a culture of the single basidiospore isolate B-3502 (*a* mating type, serotype D) obtained from a cross between a type strain of *Filobasidiella neoformans*, NIH 12 (serotype D), and an isolate NIH 433 (serotype D) from Danish pigeon-droppings. The culture conditions and procedure for isolation of the polysaccharide were as already described[1]. Purified polysaccharide was obtained by chromatography on a column (2.5 × 30 cm) of DEAE-

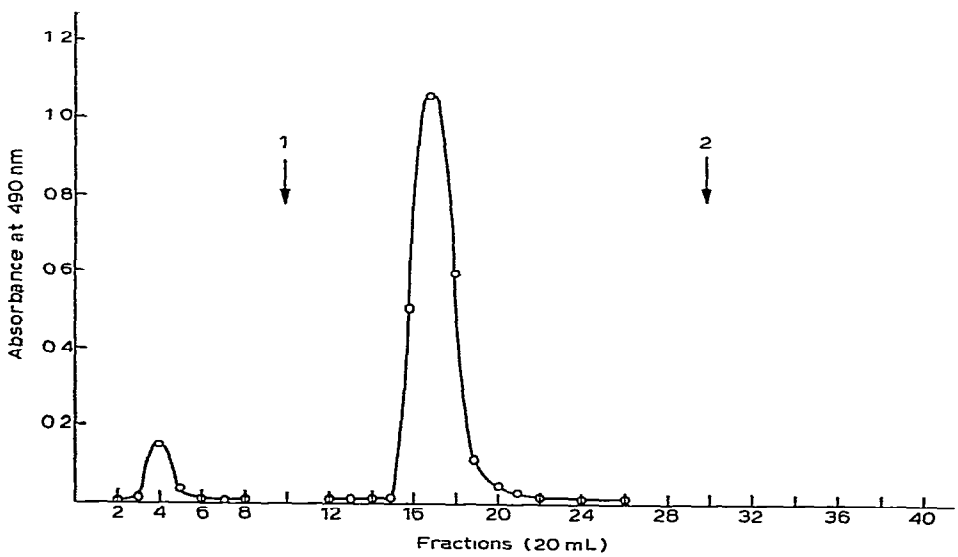

Fig 1. Chromatography of the native polysaccharide of *C. neoformans* serotype D on a column (2.5 × 30 cm) of DEAE-cellulose, using 0.01M phosphate buffer, pH 7.3. [Arrows indicate the addition of salt to the buffer to make it (*1*) 0.2M NaCl, and (*2*) 1.0M NaCl, respectively.]

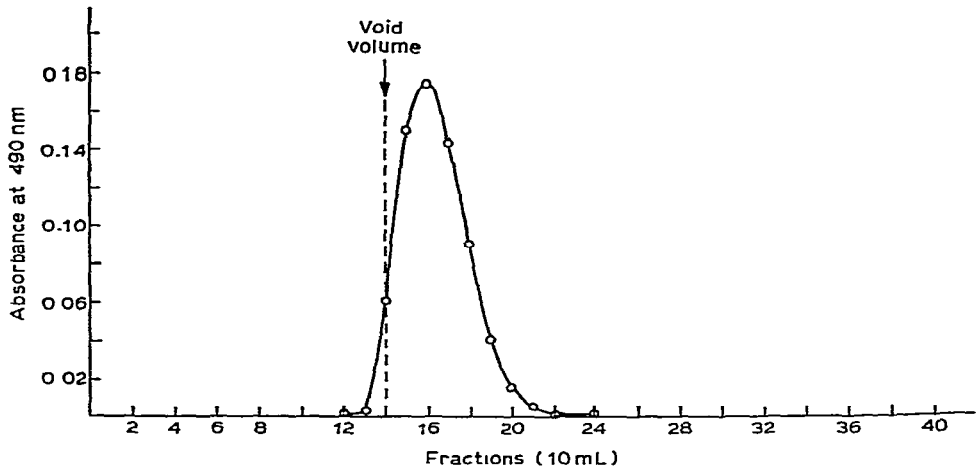

Fig. 2. Chromatography of the purified polysaccharide of *C. neoformans* serotype D on a column (2.5 × 75 cm) of Sepharose 6B, using as the eluant 0.01M phosphate buffer, pH 7.3, containing 0.15M NaCl. (The arrow indicates the void volume.)

cellulose DE-52 in the phosphate form. A neutral polysaccharide, constituting ~5–10% of the load, was first eluted with 0.01M phosphate buffer, pH 7.3. The major polysaccharide (~80%) was eluted with the same buffer but containing 0.2M NaCl (see Fig. 1). The material was recovered by dialysis against distilled water, and freeze-dried. The purified polysaccharide gave a single band in immunoelectrophoresis, and a symmetrical peak in chromatography on a column (2.5 × 75 cm) of Sepharose 6B, as shown in Fig. 2.

Chromatography. — Paper chromatography was performed by the descending method, using Whatman Nos. 1 and 3 MM paper. The solvent systems used were (*A*) 10:4:3 (v/v) ethyl acetate–pyridine–water, and (*B*) 18:3:1:4 (v/v) ethyl acetate–acetic acid–formic acid–water. The sugars were detected either by using the alkaline silver nitrate reagent[13] or by spraying with a solution of *p*-anisidine hydrochloride in 1-butanol[14].

Column chromatography was performed by using a column (2.5 × 30 cm) of DEAE-cellulose (Whatman DE-52) ion-exchange resin in the phosphate form. Gel filtration was performed in a column (2.5 × 75 cm) of Sepharose 6B (Pharmacia Fine Chemicals, Uppsala, Sweden), using as the eluant 0.01M phosphate buffer, pH 7.3, containing 0.15M NaCl. The column fractions were tested for reducing sugar content by means of the phenol–sulfuric acid reagent[15].

Gas–liquid chromatography (g.l.c.) was performed in a Finnigan-9500 gas–liquid chromatograph with a flame-ionization detector, using glass columns packed with (*a*) 3% of ECNSS-M on Gas Chrom Q (100–120 mesh) at 170° for the detection of methylated alditol acetates, and 190° for alditol acetate derivatives of free sugars; (*b*) 3% of NPGS on Gas Chrom Q (100–120 mesh) at 120° for estimation of *O*-acetyl content; and (*c*) 3% of OV-225 on Gas Chrom Q (100–120 mesh) at 170° for methylated alditol acetates.

Gas–liquid chromatography–mass spectrometry (g.l.c.–m.s.) was performed on an LKB-Bromma 2091 gas–liquid chromatograph–mass spectrometer operated at 70 eV, using a capillary column of SE-30.

Hydrolysis studies. — Complete hydrolysis of the polysaccharide was effected by heating with 0.5M aqueous sulfuric acid in sealed tubes for 16 h at 100°. Partial hydrolysis with acid was performed by heating with 0.5M aqueous hydrochloric acid for various lengths of time at 100°. It was found that heating for 4 h gave the optimal yield of oligosaccharides. Quantitative estimation of the component sugars was performed by g.l.c. of the derived alditol acetates, using inositol as the internal standard. Relative ratios were obtained by cutting out the peak areas and weighing the excised pieces.

In one experiment, the purified polysaccharide (200 mg) was taken up in 0.5M HCl (50 mL) and boiled under reflux for 4 h at 100°. The solution was cooled, made neutral with powdered silver carbonate, de-ionized with cation-exchange resin, concentrated to a small volume, and passed through a small column of Amberlite IRA-400 resin in the acetate form. The neutral sugars were eluted with water (40 mL), and the acidic sugars, with M formic acid (40 mL). The acidic fraction was fractionated on a column (1.6 × 62 cm) of Sephadex G-15 by elution with 0.05M pyridine acetate buffer, pH 5.4. Acidic oligosaccharides were further purified by paper chromatography on Whatman No. 3 MM paper, using solvent *B*. Thus obtained, the purified oligosaccharide **1** (10 mg) had $[\alpha]_{589}^{20}$ −33° (*c* 1.0, water), and oligosaccharide **2** (3 mg) had $[\sigma]_{589}^{20}$ −4° (*c* 0.3, water). On hydrolysis, oligosaccharide **1** gave mannose and glucuronic acid in the ratio of 1:1. Borohydride reduction of **1**, and hydrolysis of the product, gave glucuronic acid and mannitol.

Methylation. — The polysaccharide was methylated by the Hakomori method[16], and the product recovered by dialysis and freeze-drying. The methylated polysaccharide was heated with 90% formic acid for 1 h at 100°, and then hydrolyzed with 0.15M sulfuric acid for 16 h at 100°. The hydrolyzate was converted into alditol acetates, and these were analyzed by g.l.c. and g.l.c.–m.s.[17,18]. The methylated polysaccharide was carboxyl-reduced with lithium aluminum deuteride in tetrahydrofuran.

Chromium trioxide oxidation. — Oxidation of the acetylated polysaccharide with chromium trioxide was performed according to Lindberg *et al.*[19]. Acetylated polysaccharide (13 mg) was taken up in glacial acetic acid (3 mL), and stirred at room temperature until dissolved (2 h). Part (1 mL) of this solution was kept as a control. The rest was treated with dry chromium trioxide (200 mg), and the mixture was sonicated in an ultrasonic bath for 2 h at 50°. The control sample was treated similarly, but without adding the chromium trioxide. The reaction product was recovered free from the reagent by chromatography on a column (1 × 50 cm) of Sephadex LH-20 (Pharmacia Fine Chemicals), using acetone as the eluant, and was *O*-deacetylated with 0.1M sodium methoxide in methanol, and the product analyzed by hydrolysis and conversion of the products into the alditol acetates.

Periodate oxidation. — A solution of the polysaccharide in 0.02M sodium metaperiodate solution was kept in the dark at room temperature. Aliquots were with-

drawn at intervals, and the consumption of periodate was measured by recording the optical absorbance[20] at 223 nm.

Smith degradation. — Purified polysaccharide from *C. neoformans* serotype D (96 mg) was taken up in water (50 mL), and the suspension was stirred until it gave a clear solution. Sodium metaperiodate solution (50 mL; 0.05M) was then added with stirring, and the mixture was kept in the dark for 24 h at room temperature. The excess of periodate was decomposed by adding the calculated amount of ethylene glycol, powdered sodium borohydride (200 mg) was added, and the mixture was kept for 7 h at room temperature; the excess of borohydride was neutralized with M acetic acid, and the mixture dialyzed against distilled water. The dialyzed solution (120 mL) was concentrated to 40 mL in a rotary evaporator, and the solution was made 0.5M with hydrochloric acid. After 7 h at room temperature, the acid was neutralized with 10M sodium hydroxide solution, and the Smith-degraded polysaccharide was recovered by dialysis and freeze-drying; yield 45.7 mg; $[\alpha]_{589}^{20}$ +115° (*c* 0.5, H_2O). The Smith-degraded polysaccharide was methylated by the Hakomori method[16].

RESULTS AND DISCUSSION

The purified polysaccharide of *C. neoformans* serotype D was obtained by ion-exchange chromatography on DEAE-cellulose, and gave a homogeneous peak in gel chromatography on Sepharose 6B, as shown in Fig. 2. The K_{av} was 0.09, indicating a very high molecular weight (~8×10^5). The purified polysaccharide had $[\alpha]_{589}^{20}$ +32° (*c* 0.2, H_2O), and gave a viscous solution at a concentration of 0.3% in water. Elemental analysis showed C, 39.8; H, 5.59; N, 0.23; and P, 0.37%. Estimation of *O*-acetyl by the method of Bethge and Lindstrom[21] showed the presence of 10.3% of *O*-acetyl. The polysaccharide gave a single band in immunoelectrophoresis (see Fig. 3), and moved towards the anode. The ^{1}H.n.m.r. spectrum of the polysaccharide showed a

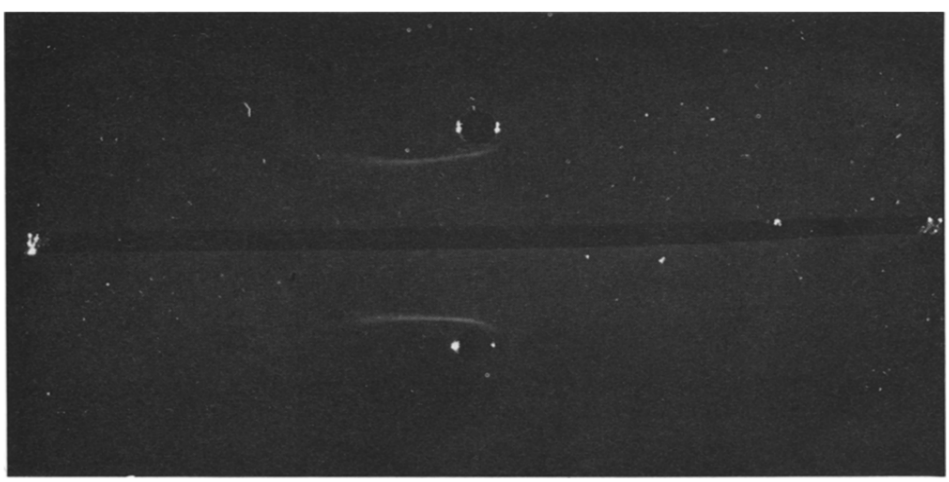

Fig. 3. Immunoelectrophoresis of purified polysaccharide of *C. neoformans* serotype D in 0.1M Tris acetate buffer, pH 8.3, at a constant 6 V. cm^{-1} for 1.5 h. (The anode is on the left.)

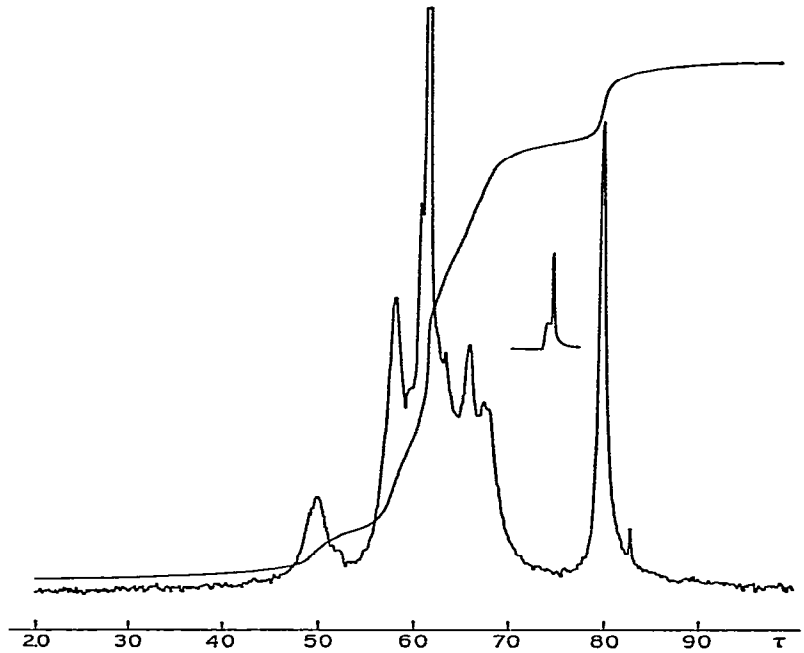

Fig. 4. ^{1}H-N m.r. spectrum of purified polysaccharide of *C. neoformans* serotype D in D_2O at 95°.

sharp *O*-acetyl peak at τ 8.0, and a single, broad, anomeric peak at τ 5.0. The ratio of protons in the acetyl: anomeric peaks was ~1.8:1.0 (see Fig. 4).

Complete hydrolysis of the polysaccharide, followed by chromatography in solvents *A* and *B*, showed the presence of three sugars, having mobilities identical to those of D-mannose, D-xylose, and D-glucuronic acid. The identity of the sugars was confirmed by their conversion into alditol acetates, followed by g.l.c. in column (*a*), using inositol hexaacetate as the internal standard[22]. The presence of glucuronic acid was confirmed by analysis of the carboxyl-reduced polysaccharide[12], when glucose was obtained (instead of glucuronic acid). Quantitative estimation of the sugars showed the presence of mannose, xylose, and glucuronic acid in the ratios of ~3:1:1. Hydrolysis of the polysaccharide, followed by isolation of the individual sugars by preparative paper-chromatography and measurement of their optical rotations, showed that all of the sugars had the D configuration.

Partial hydrolysis of the purified polysaccharide (200 mg) with 0.5M HCl for 4 h at 100° gave only small amounts of oligosaccharides, although this was found to be the optimal condition for oligosaccharide formation. Two acidic oligosaccharides were isolated, in yields of 10 mg and 3 mg, respectively. Oligosaccharide **1** was identified as 2-*O*-(*β*-D-glucopyranosyluronic acid)-D-mannose by hydrolysis, by borohydride reduction followed by hydrolysis, and by methylation analysis. The optical rotation of oligosaccharide **1**, $[\alpha]_{589}^{20}$ −33° (*c* 1.0, water), indicated that the D-glucuronic acid was linked *β* to the D-mannose reducing unit; this was further confirmed by the release of D-glucuronic acid on treatment with *β*-D-glucosiduronase (Boehringer–Mannheim).

Oligosaccharide **2** was a trisaccharide composed of two mannose residues and one glucuronic acid residue, with a mannose residue as the reducing residue. The optical rotation, —4°, also indicated a β-D-linked glucuronic acid unit. The optical rotation would be expected to be less negative than that of **1**, as, by analysis by oxidation with chromium trioxide, all of the D-mannose residues were shown to be α-D-linked.

Chromium trioxide oxidation[23] of the polysaccharide resulted in the loss of 80% of the D-xylose residues (in 2 h), showing that these units are β-D-linked in the polysaccharide, and that the D-mannose is α-D-linked.

Periodate oxidation of the polysaccharide resulted in the uptake of 1.0 mol of periodate per mol (constant after 24 h; see Fig. 5). Periodate oxidation followed by

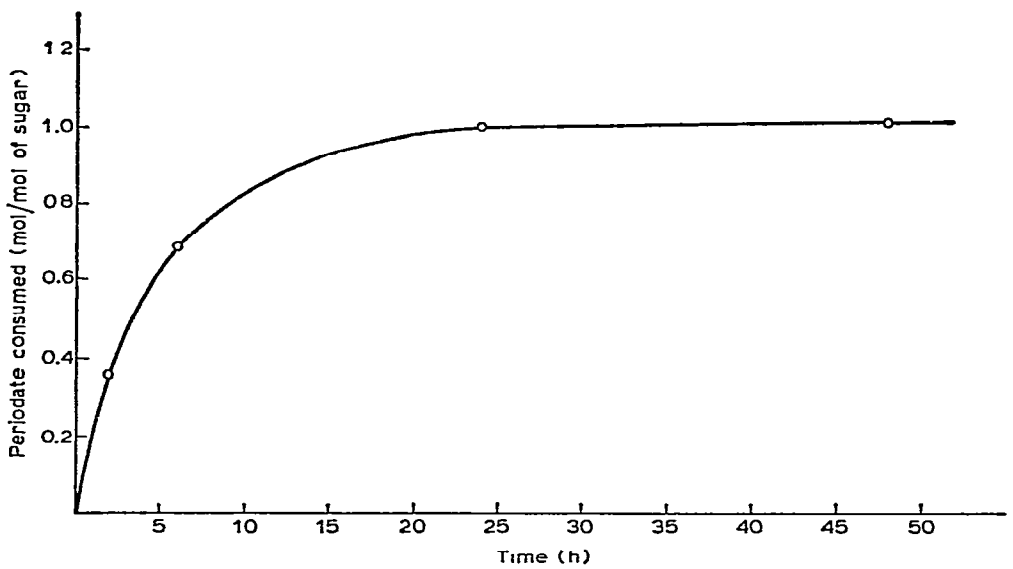

Fig. 5. Periodate oxidation of the capsular polysaccharide of *C. neoformans* serotype D. (The consumption of periodate is based on an average molecular weight of 180.)

borohydride reduction and mild hydrolysis (Smith degradation[24]) gave rise to a periodate-resistant polysaccharide which was recovered by dialysis and freeze-drying. This material had $[\alpha]_{589}^{20}$ +115° (*c* 0.5, H_2O), and hydrolysis thereof gave only mannose. Methylation, followed by hydrolysis, conversion into alditol acetates, and analysis by g.l.c. and g.l.c.–m.s. showed the presence of 2,4,6-tri-*O*-methylmannose as the major component (93%), indicating that the periodate-resistant backbone is a (1 → 3)-linked mannan. However, there was also a minor component (7%) which was found to be 4,6-di-*O*-methylmannose. If demethylation is ignored, this indicates that 1 in 15 mannosyl residues in the mannan backbone carries a branch at O-2.

The following structural formula is proposed for the capsular polysaccharide here investigated.

—D-Man*p*-(1 → 3)-α-D-Man*p*-(1 → 3)-α-D-Man*p*-(1 → 3)-
2 2
↑ ↑
1 1
β-D-Xyl*p* β-D-Glc*p*A

Methylation of the polysaccharide, followed by hydrolysis, and analysis of the sugars as alditol acetates by g.l.c. and g.l.c.–m.s., showed the presence of 2,3,4-tri-*O*-methylxylose, 2,4,6-tri-*O*-methylmannose, 4,6-di-*O*-methylmannose, and 6-*O*-methylmannose in the molar ratios of ~2:2:3.0 7. The (glucosyluronic acid)–mannose linkage would be expected to be resistant to hydrolysis, and it is not seen in the analysis. Therefore, the amount of di-*O*-methylmannose from I (see Table I) will be below the

TABLE I

METHYLATION ANALYSIS OF THE CAPSULAR POLYSACCHARIDE OF *C. neoformans* SEROTYPE D[a]

Methylated sugars[b]	*T*[c]	*Sample*[d]		
		I (*Moles*)	*II*	*III*
2,3,4,6-Me_4-Glc	1.00		1 3	
2,3,4-Me_3-Xyl	0 66	2	1	
2,4,6-Me_3-Man	2.12	2	1.7	4 9
4,6-Me_2-Man	3.34	3	2	0.36
6-Me-Man	4.48	0.7	0.3	

[a]All sugars were analyzed as methylated alditol acetates, using column (*a*) at 170°. [b]2,3,4,6-Me_4-Glc = 2,3,4,6-tetra-*O*-methylglucose, etc. [c]Retention times are relative to that of 1,5 di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol as unity. [d](I) Methylated polysaccharide type D; (II) methylated, carboxyl-reduced polysaccharide type D; (III) methylated, Smith-degraded polysaccharide type D

theoretical ratio, which, for the hydrolysis of I is tri-*O*-methylxylose:tri-*O*-methylmannose:di-*O*-methylmannose = 1:1·2, and it may be seen that this low ratio was found.

Methylation analysis of the carboxyl-reduced polysaccharide showed the presence of 2,3,4,6-tetra-*O*-methylglucose, in addition to the preceding sugars, as shown in Table I. The ratio of methylated glucose to methylated mannose is also ~1:3. These results show that both the glucuronic acid and the xylose units are present as non-reducing end-groups attached to O-2 of mannose residues, but ~1 out of every 3 mannose units is not substituted. The amount of tri-*O*-methylmannose obtained in the hydrolysis of II (see Table I) was higher than expected, but no explanation is yet at hand.

The sequence of D-glucosyluronic acid and D-xylosyl groups in our proposed structure can be interchanged without affecting the results. The presence of a small proportion of 6-*O*-methylmannose (see Table I) indicates that mannose units are substituted on both O-2 and O-4; this was confirmed by the results of the Smith degradation, which showed that 1 out of every 15 mannose units carries a branch on O-2.

Therefore, the 6-*O*-methylmannose obtained from I (Table I) arises from the branching mannose units bearing a substituent on O-4.

The estimation of *O*-acetyl content of the polysaccharide by the method of Bethge and Lindstrom[21] showed the presence of 10.3% of *O*-acetyl. The ^{1}H-n.m.r. spectrum showed a sharp acetate peak at τ 8.0. The ratio of protons in the acetate peak and the anomeric peak was ~9:5, indicating the presence of three *O*-acetyl groups per five sugar residues. Attempts at locating the *O*-acetyl groups by protecting the free hydroxyl groups with methyl vinyl ether, followed by methylation analysis[25], failed to give interpretable results. However, the periodate oxidation results show that the xylosyl or glucosyluronic acid groups could not be substituted with *O*-acetyl groups, as, in that case, there would be a much lower consumption of periodate. Therefore, the *O*-acetyl groups must be attached to the mannose residues. As the polysaccharide is heavily *O*-acetylated, these acetyl groups may contribute to the antigenic specificity; this was found to be the case, by a precipitin test in agar gel (see Fig. 6).

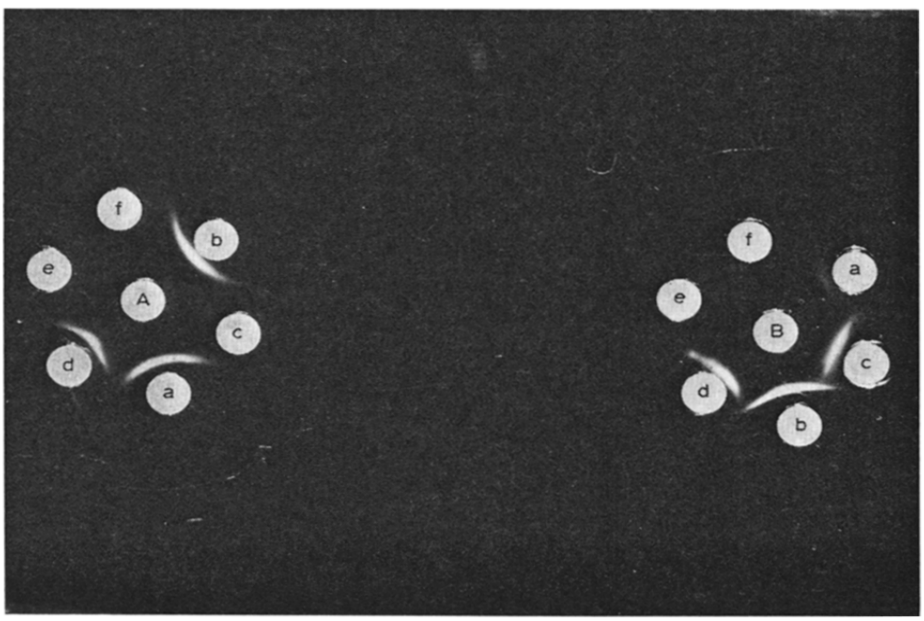

Fig. 6. Immunodiffusion test in agar gel, using (A) rabbit antiserum to *C. neoformans* serotype C cells and (B) rabbit antiserum to *C. neoformans* serotype D cells. [The antigens used in the peripheral cups were (a) purified polysaccharide serotype C; (b) purified polysaccharide serotype D; (c) carboxyl-reduced polysaccharide serotype D; (d) *O*-deacetylated polysaccharide serotype D; (e) Smith-degraded polysaccharide serotype D, and (f) carboxyl-reduced, *O*-deacetylated polysaccharide serotype D]

An immunodiffusion test[26] in agar gel, using rabbit antisera to whole cells of *C. neoformans* serotypes C and D (see Fig. 6), showed that the sera give sharp bands against type-specific polysaccharides. The anti-type D serum was only weakly cross-reactive with type C polysaccharide, probably because C polysaccharide has few, if

any, *O*-acetyl groups[1]. However, anti-type C serum reacted strongly with type D polysaccharide. Carboxyl-reduced polysaccharide type D reacted very weakly with anti-type C serum, showing that the carboxyl groups are strong determinants for this antiserum. Carboxyl-reduced and *O*-deacetylated polysaccharide type D gave a very weak or no precipitin band against anti-type D serum, showing that both the carboxyl and the *O*-acetyl groups are important determinants of antigenic specificity in this polysaccharide. Smith-degraded polysaccharide failed to show any precipitin band against either serum, indicating the absence of precipitating antibody against the D-mannan backbone.

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