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CAPSULAR POLYSACCHARIDES FROM A PARENT STRAIN AND FROM A POSSIBLE, MUTANT STRAIN OF *Cryptococcus neoformans* SEROTYPE A

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ABSTRACT

The capsular polysaccharides from *Cryptococcus neoformans* serotype A and from a possible, mutant strain thereof have been structurally analyzed. The latter material is similar to the capsular antigen of *C. neoformans* serotype D.

INTRODUCTION

Two serotypic variations (serotypes A and D) are known among the isolates of *Cryptococcus neoformans*[1]. Most of the cases of cryptococcosis in the U.S.A. are caused by serotype A strains, the frequency of serotype D among clinical isolates being[2] only ~5%. The serotypes are defined by the antigenic determinants of the extracellular polysaccharide, and the chemical structures both of serotypes A and D have been elucidated, that of the former by Cherniak *et al.*[3] and Merrifield and Stephen[4], and that of the latter by us[5]. The structure reported by Cherniak *et al.*[3] for the polysaccharide from serotype A differed from that educed by Merrifield and Stephen[4], the former workers[3] finding a structure that was actually quite similar to that reported by us[5] for serotype D.

In the laboratory of one of us (K. J. K.-C.), a strain of *C. neoformans* type A (strain no. 371) that had been stored as a stock culture for ~15 years showed, on subculture, colonies that had two different characteristics. Upon cloning, two stable populations were obtained, namely, serotype A and serotype D. The original isolate was obtained from cuckoo droppings collected in Thailand, and had been subcultured many times before it was serotyped as A. (It had earlier been found by Bennett *et al.*[2] that, although serotypes of each isolate are stable, serotypes A and D can form a continuous spectrum of antigenic variation. Although most isolates are clearly A or D, some are of intermediate nature, and type as A, D, or A/D, depending on the antisera used and the number of absorptions made.) Two explanations are possible for the apparent change from A → (A + D). One is that the isolate was originally a mixture of serotypes A and D, but that the D-cells were not detected during the typing. The other is that the original isolate was pure A-type, but that some cells mutated to serotype D during maintenance. In view of the discrepancy in the structures

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reported[3,4] for serotype A polysaccharide, we decided to study the structures of the capsular polysaccharide from serotype A and D cloned from the original culture, and now report the results of this study.

EXPERIMENTAL

General methods. — Evaporations were performed in a rotary evaporator at or below 40°. Optical rotations were recorded with a Perkin–Elmer 141 polarimeter at 20 ±1°, using sodium light (589 nm). ^{13}C-N.m.r. spectra were recorded at 67.89 MHz with an NIH-modified spectrometer having a Burker magnet and equipped with a Nicolet model 1080 computer of maximum 34k data memory. Spectra were collected (with complete, proton-noise decoupling) by using a 15-kHz sweep-width and 16k data points, at probe temperatures of 70 ±2°. 1,4-Dioxane was used as the internal reference, at 69.16 p.p.m. from tetramethylsilane.

Carboxyl-reduced polysaccharides were prepared by the method of Taylor and Conrad[6]; two treatments with the reagents were needed for complete reduction. *O*-Deacetylated polysaccharide was prepared as already described[7]. Double diffusion in agar gel was performed by the method of Ouchterlony[8]. Rabbit antisera raised against whole cells of *C. neoformans* serotype A and serotype D (kindly presented by Dr. J. E. Bennett) were used.

Preparation of purified polysaccharide. — *Cryptococcus neoformans* serotype A (strain No. 371) was cloned into two different strains, 371-a and 371-3. These were cultured, and crude polysaccharides were isolated by a method already described[9]. Polysaccharide 371-3 was further purified by chromatography on a column (2.5 × 74 cm) of Sepharose 6B, using phosphate-buffered saline (PBS; 0.01M phosphate, 0.15M NaCl, pH 7.4) as the eluant. The major-peak material (see Fig. 1) was isolated, and rechromatographed on a column (1.6 × 65 cm) of DEAE-cellulose by using

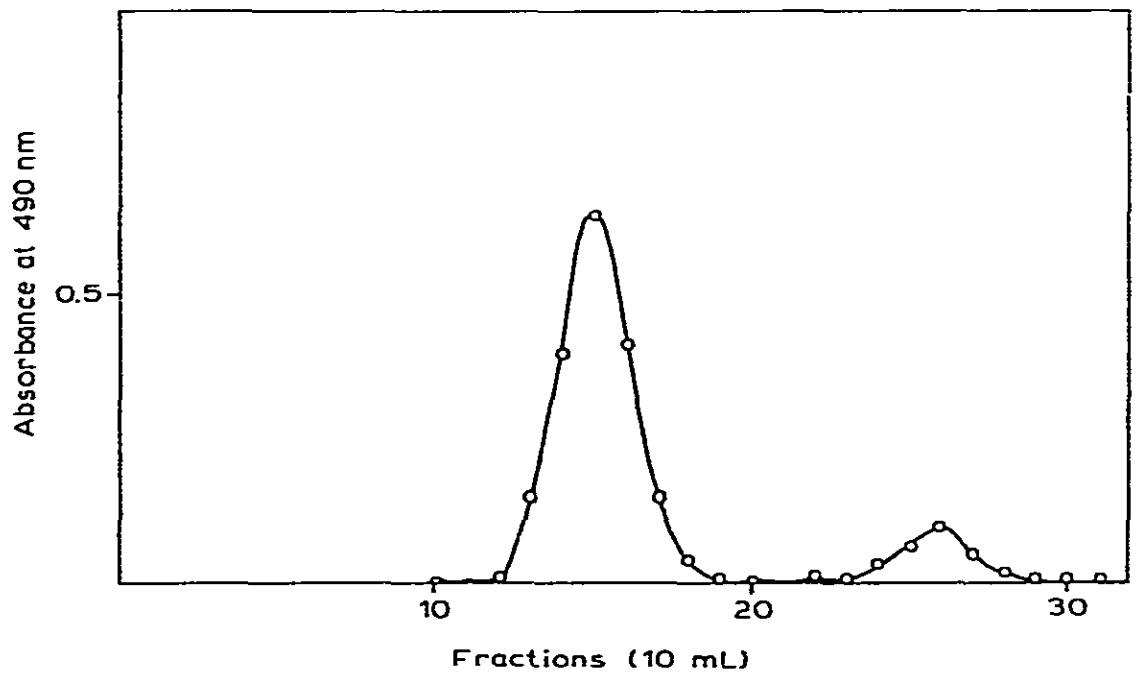


Fig. 1. Chromatography of the capsular material 371-3 on a column (2.5 × 75 cm) of Sepharose 6B, using PBS, pH 7.4, as the eluant.

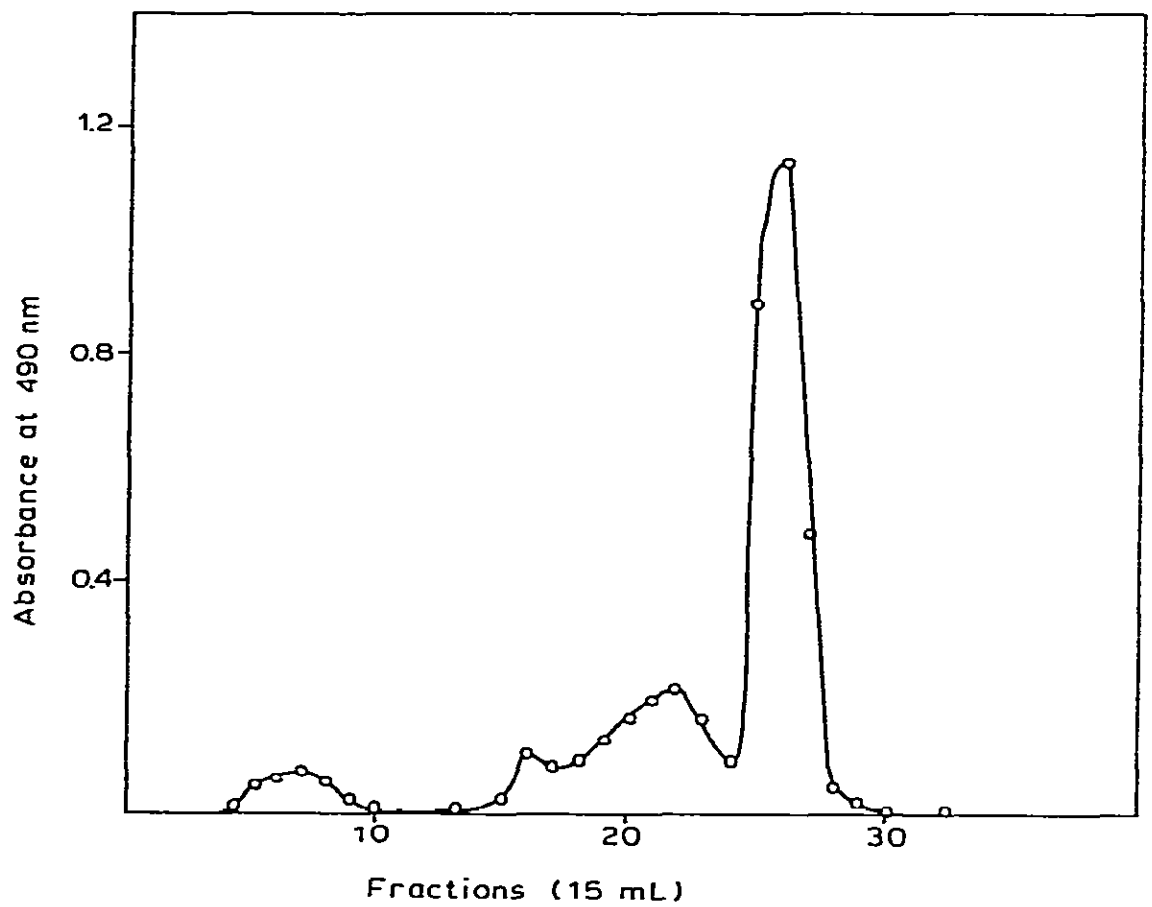


Fig. 2. Chromatography of the major polysaccharide fraction (from the Sepharose 6B column) on a column (1.6 × 65 cm) of DEAE-cellulose, using 0.01M phosphate buffer, pH 7.3, containing a gradient of 0 → 1.0M NaCl.

gradient elution with 0.01M phosphate buffer, pH 7.4, from 0 to 1.0M in NaCl. One major peak was obtained at a salt concentration of 0.28–0.35M (see Fig. 2). The material in the major peak was isolated after extensive dialysis, and was used for the structural investigation. On the other hand, polysaccharide 371-a gave a single peak on both of these columns, and was therefore used without further purification.

Chromatography. — Paper chromatography was performed by the descending method, using Whatman No. 1 paper. The solvent systems used were (*A*) 6:4:3 (v/v) 1-butanol–pyridine–water, (*B*) 18:3:1:4 (v/v) ethyl acetate–acetic acid–formic acid–water, and (*C*) 10:4:3 (v/v) ethyl acetate–pyridine–water. The sugars were detected by spraying with alkaline silver nitrate[10].

Column chromatography was performed by using a column (2.5 × 74 cm) of Sepharose 6B (Pharmacia Fine Chemicals, Uppsala, Sweden), with elution with PBS, pH 7.4. Ion-exchange chromatography was conducted on a column (1.6 × 65 cm) of DEAE-cellulose (Whatman, DE-52) in the phosphate form. The column was first equilibrated with 0.01M phosphate buffer, pH 7.4, before adding the sample.

Gas–liquid chromatography (g.l.c.) was performed in a Finnegan 9500 gas–liquid chromatograph equipped with a flame-ionization detector and an HP-3380A (Hewlett–Packard) digital integrator. The glass columns used were packed with (*a*) 3% of ECNSS-M on Gas Chrom Q (100–120 mesh), (*b*) 3% of OV-225 on Gas Chrom Q (100–120 mesh), and (*c*) 3% of SP-2340 on Supelcoport (100–120 mesh). Gas–liquid chromatography–mass spectrometry (g.l.c.–m.s.) was performed with a

V.G. micromass 7070F spectrometer (70 eV) connected to a Perkin–Elmer Sigma 3 gas chromatograph.

Methylation. — The polysaccharides were methylated with the Hakomori reagents[11] and, after extensive dialysis against distilled water, the products were recovered by freeze-drying. In one experiment, the methylated polysaccharide was remethylated by the Kuhn method[12]. The methylated polysaccharides were hydrolyzed, the liberated sugars converted into their alditol acetates, and these analyzed by g.l.c. and g.l.c.–m.s.[13,14].

Alkaline β-elimination. — Alkaline degradation of the methylated polysaccharide was performed by the method of Lindberg *et al.*[15], as modified by Aspinall and Rosell[16]. The alkali-degraded polysaccharide was recovered by freeze-drying after extensive dialysis against distilled water, hydrolyzed, the products reduced, and the alditol derivatives analyzed as partially methylated acetates.

Smith degradation. — A solution of the polysaccharide (56 mg) in water (25 mL) was treated with a 0.04M solution (25 mL) of sodium metaperiodate, and the mixture was kept in the dark for 72 h at room temperature. The excess of periodate was then decomposed by addition of ethylene glycol. Sodium borohydride (100 mg) was now added, and the mixture was kept for 20 h at room temperature, and dialyzed against distilled water; to the dialyzed solution (55 mL) was added 6M HCl to a final concentration of 0.5M, and the mixture was kept for 7 h at room temperature. The acid was then neutralized with 5M NaOH (to a pH of 6.0), and the solution of degraded polysaccharide was dialyzed and freeze-dried; yield 24 mg.

RESULTS AND DISCUSSION

Polysaccharide 371-a gave a single peak in ion-exchange chromatography (see Fig. 3) on DEAE-cellulose and in gel filtration on Sepharose 6B. It had $[\alpha]_{589}^{20}$

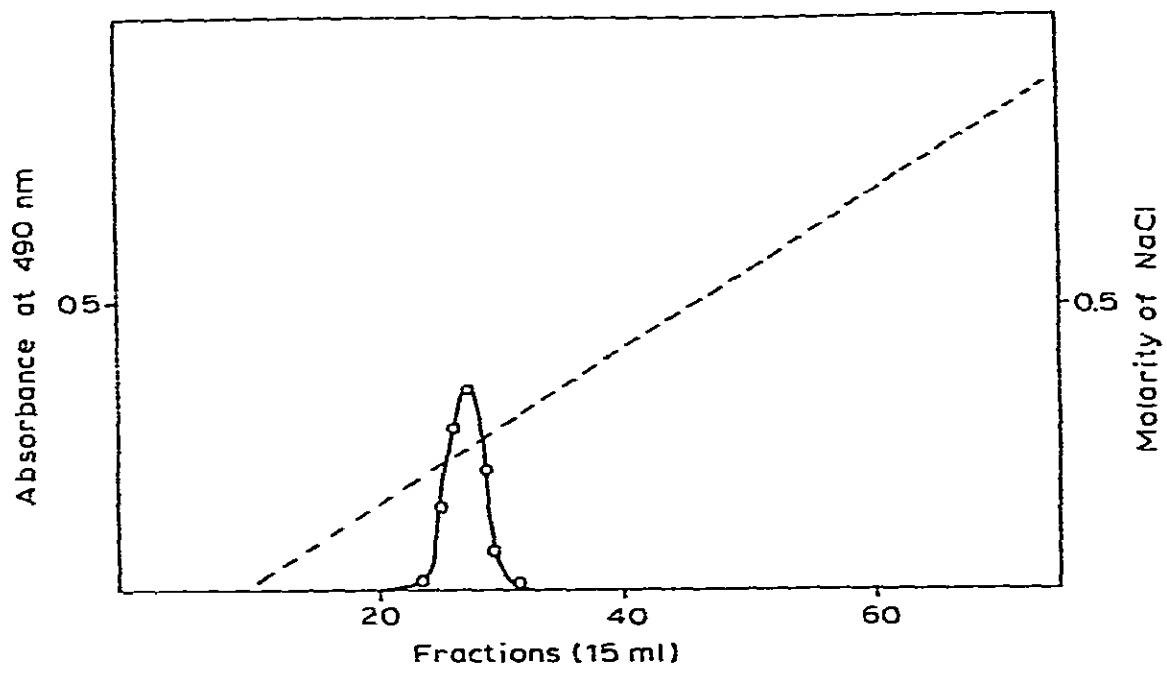


Fig. 3. Chromatography of the capsular material 371-a on a column (1.6 × 60 cm) of DEAE-cellulose, using 0.01M phosphate buffer, pH 7.4, containing a gradient of 0 → 0.9M NaCl.

+26° (*c* 0.25, water). Hydrolysis of this polysaccharide with 0.5M sulfuric acid for 16 h at 100°, followed by paper chromatography in solvents *B* and *C*, showed the presence of xylose, mannose, and glucuronic acid as component sugars. Hydrolysis of the carboxyl-reduced polysaccharide, followed by analysis by g.l.c. as alditol acetates, using column (*a*), showed the presence of mannose, xylose, and glucose in the molar ratios of ~22:8:5. Estimation of glucuronic acid in the polysaccharide by the *m*-hydroxybiphenyl reagent[17] showed 14.3% as glucuronic acid. Methylation of the carboxyl-reduced polysaccharide, followed by hydrolysis and analysis by g.l.c. and g.l.c.–m.s., showed the presence of 2,3,4-tri-*O*-methylxylose, 2,3,4,6-tetra-*O*-methylglucose, 2,4,6-tri-*O*-methylmannose, 4,6-di-*O*-methylmannose, and 6-*O*-methylmannose in the molar ratios of ~18:1:15:20:3. Methylation of the purified polysaccharide, followed by reduction with lithium aluminum deuteride, and analysis as before, showed the presence of methylated sugars in the same ratios as just given, except that 2,3,4-tri-*O*-methylglucose was obtained, instead of 2,3,4,6-tetra-*O*-methylglucose.

Methylated, Smith-degraded polysaccharide was hydrolyzed, and analysis of the products as their alditol acetates showed the presence of 2,4,6-tri-*O*-methylmannose and 4,6-di-*O*-methylmannose in the ratio of 97:3. On similar analysis, the alkali-degraded, remethylated polysaccharide showed the presence of 2,3,4-tri-*O*-methylxylose, 2,4,6-tri-*O*-methylmannose, and 4,6-di-*O*-methylmannose in the molar ratios of ~1:1.9:1.6 (see Table I). The absence of any monomethylmannose from this sample is significant. The Smith-degraded polysaccharide was insoluble in water, but could be dissolved in aqueous dimethyl sulfoxide; $[\alpha]_{589}^{20}$ +120° (*c* 0.25, 2:1 Me_2SO–H_2O).

Polysaccharide 371-3 was heterogeneous, and was purified by chromatography on Sepharose 6B (see Fig. 2), followed by ion-exchange chromatography on DEAE-

TABLE I

METHYLATION ANALYSIS OF POLYSACCHARIDE 371-a

Methylated sugars[a] *(alditol acetates)*	T[b]	*Molar ratios*[c]		
		I	*II*	*III*
2,3,4-Me_3-Xyl	0.66	1.8	1.0	—
2,3,4,6-Me_4-Glc	1.00	1.0	—	—
2,4,6-Me_3-Man	2.08	1.5	1.94	9.7
2,3,4-Me_3-Glc	2.43	—	—	—
4,6-Me_2-Man	3.34	2.0	1.6	0.3
6-Me-Man	4.48	0.3	—	—

[a]2,3,4-Me_3-Xyl = 2,3,4-tri-*O*-methyl-D-xylose, etc. [b]Retention times are relative to that of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol, on column (*a*) at 170°. [c]I, Carboxyl-reduced, methylated polysaccharide; II, alkali-degraded, remethylated polysaccharide; and III, methylated, Smith-degraded polysaccharide.

TABLE II

METHYLATION ANALYSIS OF POLYSACCHARIDE 371-3

Methylated sugars[a] *(alditol acetates)*	T[b]	*Molar ratios*[c]	
		I	*II*
2,3,4-Me_3-Xyl	0.65	1.6	1.6
2,3,4,6-Me_4-Glc	1.00	1.0	—
2,4,6-Me_3Man	2.09	0.3	0.2
2,3,4-Me_3-Glc	2.42	—	1.0
4,6-Me_2-Man	3.32	2.7	2.9
6-Me-Man	4.52	0.2	—

[a]2,3,4-Me_3Xyl = 2,3,4-tri-*O*-methyl-xylose, etc. [b]Retention times are relative to that of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol as unity. [c]I, Carboxyl-reduced, methylated polysaccharide; II, carboxyl-reduced after methylation.

cellulose (see Fig. 3). The purified polysaccharide had $[\alpha]_{589}^{20}$ 0° (*c* 0.23, water). Hydrolysis of the polysaccharide with 0.5M H_2SO_4, followed by paper chromatography in solvents *A*, *B*, and *C*, showed the presence of mannose, xylose, and glucuronic acid as component sugars. Hydrolysis of the carboxyl-reduced polysaccharide, followed by analysis of the alditol acetates by g.l.c., showed the presence of mannose, xylose, and glucose in the molar ratios of ~6.6:3:2. Methylation of the carboxyl-reduced polysaccharide 371-3, followed by hydrolysis, and analysis of the partially methylated sugars as the alditol acetates by g.l.c. and g.l.c.–m.s., showed the presence of 2,3,4-tri-*O*-methylxylose, 2,3,4,6-tetra-*O*-methylglucose, 2,4,6-tri-*O*-methylmannose, 4,6-di-*O*-methylmannose, and 6-*O*-methylmannose in the molar ratios of ~16:10:3:27:2 (see Table II). Methylation of the purified polysaccharide 371-3, followed by reduction with $LiAlD_4$, and analysis as before, showed the presence of 2,3,4-tri-*O*-methylxylose, 2,3,4-tri-*O*-methylglucose, 2,4,6-tri-*O*-methylmannose, and 4,6-di-*O*-methylmannose in the molar ratios of 16:10:2:29 (see Table II). The absence of 6-*O*-methylmannose from this sample indicated complete methylation.

Smith degradation of the polysaccharide gave a degraded polymer that was insoluble in water. A solution in 2:1 Me_2SO–water had $[\alpha]_{589}^{20}$ +124° (*c* 0.25). Hydrolysis of the degraded polymer with M H_2SO_4 during 16 h at 100° gave mannose only. Methylation of the degraded polymer, followed by hydrolysis, and analysis as the alditol acetates, showed the presence of 2,4,6-tri-*O*-methylmannose only. These results showed that the backbone is a linear, (1→3)-α-linked mannan.

Figs. 4a, 4b, and 4c show the ^{13}C-n.m.r. spectra of polysaccharides 371-a and 371-3, and *O*-deacetylated polysaccharide 371-3, respectively. Although, owing to the complexity of the spectra, it was not possible to assign the resonances at δ ~102 to individual, anomeric carbon atoms, spectral differences were observed, especially in this region, supporting the differences observed in the sugar ratios and substitution

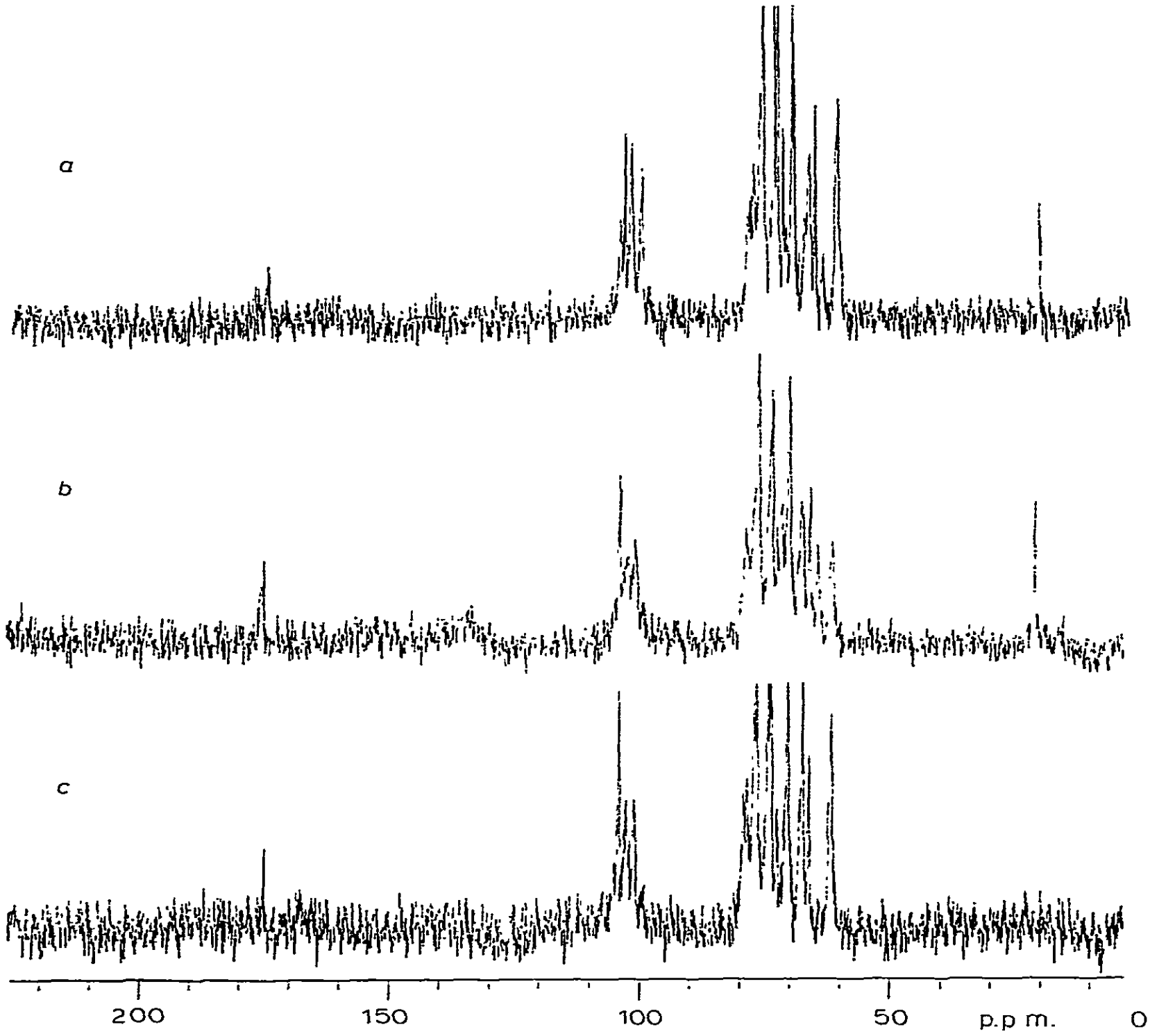


Fig. 4. Fourier-transformed, ^{13}C-n.m.r. spectra of (*a*) polysaccharide 371-a, (*b*) polysaccharide 371-3, and (*c*) *O*-deacylated polysaccharide 371-3 in D_2O (40 mg/mL) at 67.89 MHz.

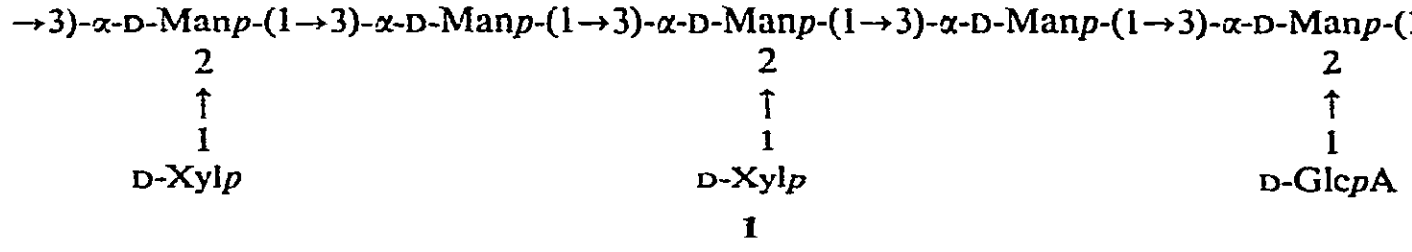
patterns as determined by structural analysis. It is obvious that both polysaccharides contain acetate groups (signal at $\delta \sim 21$) and that *O*-deacetylation effectively removed the acetate groups*.

Analysis of the sugar composition of polysaccharide 371-a showed the presence of mannose, xylose, and glucuronic acid in the molar ratios of ~4.4:1.6:1. Methylation analysis of the polysaccharide (see Table II) showed that both the xylose and

*Examination of Figs. 4b and 4c showed that there were two signals, at 62.08 and 64.9 p.p.m., and these could be assigned to C-6 of mannose in the polysaccharide 371-3. On *O*-deacetylation, the signal at 64.9 p.p.m. disappeared, with a concomitant increase in the intensity of the signal at 62.08 p.p.m. This suggests that, in polysaccharide 371-3, some of the mannose residues carry acetyl groups on O-6.

the glucuronic acid were present as nonreducing end-groups, attached to O-2 of mannose residues. The mannose residues are linked (1→3) as a linear backbone. Approximately 3 of every 8 mannose residues are unsubstituted. A very small proportion of disubstituted mannose residues (~1 out of 12) was detected, but the significance of this finding is not yet clear. The alkaline-degradation results (see Table I) showed that, on degradation, the proportion of 2,4,6-tri-*O*-methylmannose increased substantially, whereas that of 4,6-di-*O*-methylmannose decreased to the same extent. This shows that the glucuronic acid residues (which were removed during the degradation) were linked to O-2 of mannose residues bearing no other substituent.

The Smith-degradation results showed that the backbone of the polysaccharide is a linear, (1→3)-α-linked mannan. The presence of a very small proportion (~3%) of 4,6-di-*O*-methylmannose in this sample is, in our opinion, insignificant. The foregoing, structural features for the capsular polysaccharide of *C. neoformans* strain No. 371-a which has been typed here as serotype D can be represented by structural formula **1**. The D configuration of the sugars is based on our previous studies[5] on



1

the capsular polysaccharide from *C. neoformans* No. 3502 type D. The structures of these two polysaccharides are almost identical, except for the sugar ratios which, for strain No. 3502, are D-mannose, D-xylose, and D-glucuronic acid (~3:1:1), and for strain No. 371-a, are 4.4:1.6:1, but this difference is minor. The structure reported here for capsular polysaccharide 371-a is very similar to that of a capsular polysaccharide from *C. neoformans* strain No. 371 reported by Cherniak *et al.*[3]. These authors considered strain No. 371 (N.I.H.) to be serotype A, but, in view of our findings, it seems probable that their compound was a polysaccharide of serotype D (see **1**).

The capsular polysaccharide of *C. neoformans* strain No. 371-3, typed here as serotype A, had $[\alpha]_{589}^{20}$ +26°, and contained D-mannose, D-xylose, and D-glucuronic acid in the molar ratios of 3.3:1.5:1. Methylation analysis of the carboxyl-reduced polysaccharide (see Table II) showed that the D-xylose and D-glucuronic acid were both present as (nonreducing) end-groups attached to O-2 of the D-mannosyl residues. Almost all of the D-mannosyl residues carried a single substituent of either a D-xylosyl or D-glucosyluronic acid group. The very small proportion of 2,4,6-tri-*O*-methyl-D-mannose obtained indicates the presence of some (~1 in 10) unsubstituted D-mannosyl residues. The Smith-degradation results showed that the D-mannosyl residues were present as a linear, (1→3)-linked backbone. These structural features of capsular polysaccharide 371-3 can be represented by structural formula **2**, which does not show the very small proportion of unsubstituted D-mannosyl residues; it is similar to the

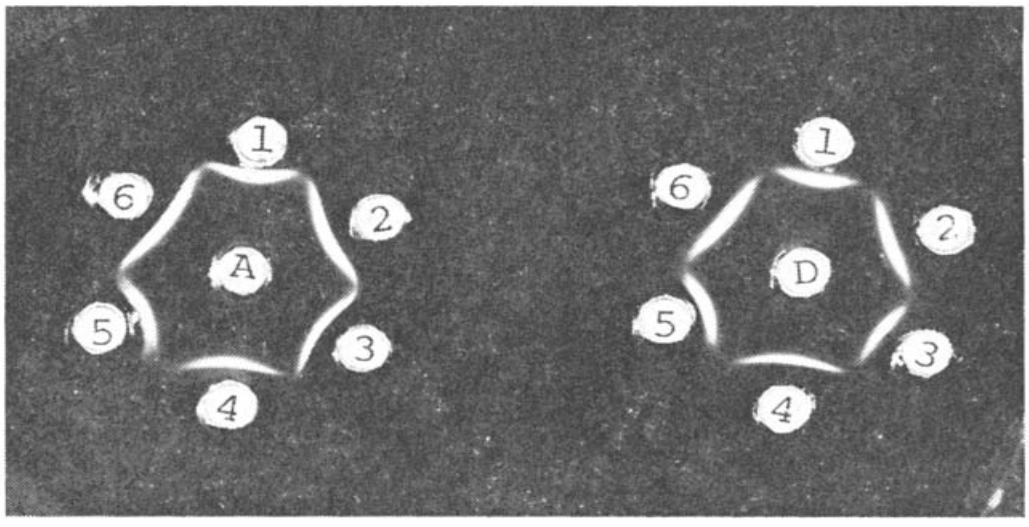


Fig. 5. Immunodiffusion test in agar gel using (A) antitype A serum and (D) antitype D serum. The antigens used are (1) native polysaccharide 371-3, (2) native polysaccharide type A, (3) purified polysaccharide 371-3, (4) purified polysaccharide No. 3502 type D, (5) native polysaccharide 371-a, and (6) native polysaccharide type A.

structure of capsular polysaccharide H obtained from a strain of *C. neoformans* serotype A by Merrifield and Stephen[4]. There are two minor differences in the sugar

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

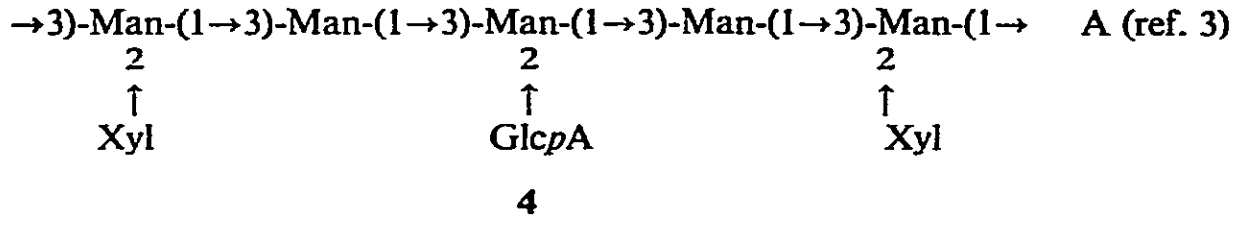
ratios, and we have no evidence for some β-linkages in the D-mannan backbone. The very small proportion of unsubstituted D-mannosyl residues detected in the polysaccharide of strain No. 371-3 was not evident in polysaccharide H.

An immunodiffusion test in agar gel (see Fig. 5) was performed with sera respectively raised against whole cells of *C. neoformans* serotype A and serotype D. Various preparations of serotype A and serotype D polysaccharides were used as precipitins. It may be seen that the polysaccharides have strong cross-reactions, and cannot be distinguished from one another by this test.

These studies, together with results from our previous studies[5,9], show some clear differences in the structures of the capsular polysaccharides from *C. neoformans* serotypes A and D, and *C. bacillisporus* serotypes[18,19] B and C, shown in formulas 3–7. One important structural feature of serotype A seems to be that all of the D-mannosyl residues in the main chain carry a single substituent, and there are very few (or no)

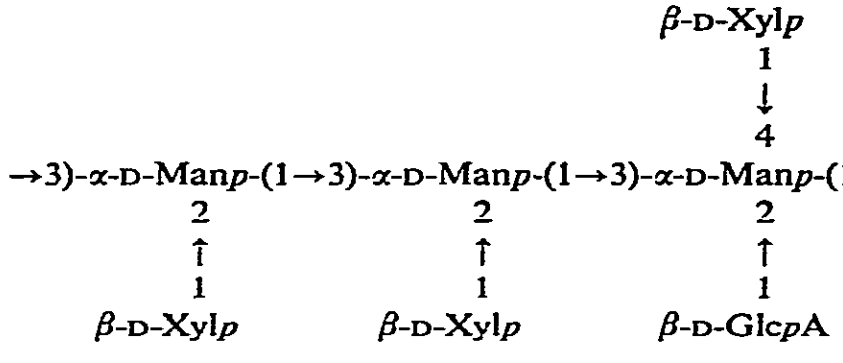
→3)-α-D-Man*p*-(1→3)-α-D-Man*p*-(1→3)-D-Man*p*-(1→
2 2 2
↑ ↑ ↑
1 1 1
β-D-GlcpA D-Xyl*p* D-Xyl*p*
3

Serotype
A (ref. 4)



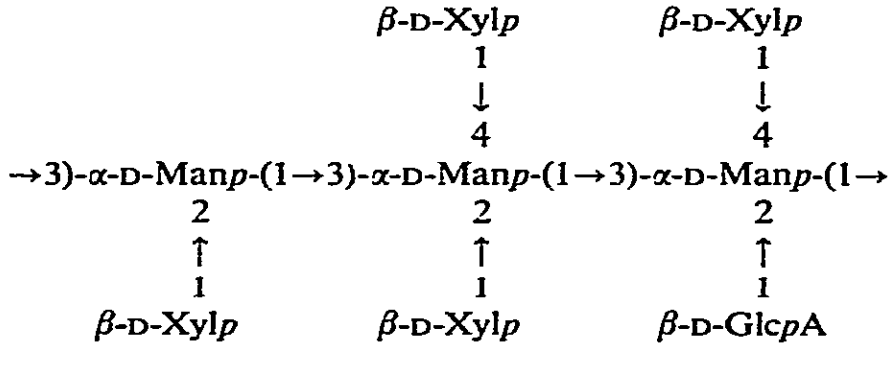
A (ref. 3)

4



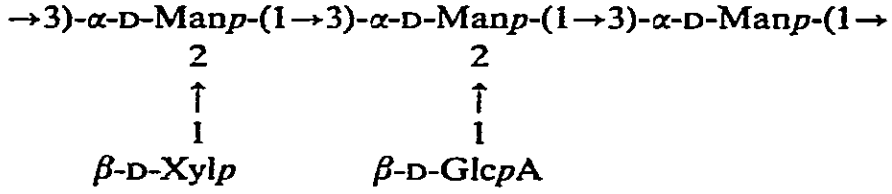
B (ref. 18)

5



C (refs. 9 and 19)

6



D (ref. 5)

7

unsubstituted D-mannosyl residues present. In contrast, the characteristic feature of serotype D polysaccharide is that it contains a substantial proportion of unsubstituted D-mannosyl residues in the main chain. In serotypes B and C, on the other hand, there are no unsubstituted D-mannosyl residues, and there are increasing proportions of disubstituted D-mannosyl residues. The finding that polysaccharide 371-3 (serotype A) contains some unsubstituted D-mannosyl residues (~1 in 10) is interesting, in that it constitutes a feature of type D polysaccharide; this supports the finding of Bennett *et al.*[2] that some strains could type as A, D, or AD.

ACKNOWLEDGMENTS

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