Polysaccharide Antigens from *Histoplasma capsulatum*.* (29010)

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Polysaccharides isolated from *Histoplasma capsulatum* have precipitin and complement-fixing activity and are capable of producing delayed hypersensitivity reactions in animals and man(1-3). Marked differences in nitrogen content and in other properties suggest that mycelial(4) and yeast phase polysaccharide(s) may differ in structure; serologic data also suggest a difference(3). An alternative explanation is that the materials used in these investigations were complex mixtures and that various preparations lacked one or more antigenic constituents, thus accounting for the diversity of reactions.

To investigate this point, methods have been developed in this laboratory for isolation and fractionation of the polysaccharides present in the culture filtrate after growth of *H. capsulatum* in the yeast phase. Results indicate that the initial polysaccharide isolated contains at least 2 antigenic components which are capable of participating in the precipitin reaction and producing a delayed hypersensitivity skin test in sensitized animals.

Materials and methods. Preparation of crude polysaccharide.† Three strains of *H. capsulatum* (no. 103, isolated from a patient at this institution; no. G-17M and 6651, obtained through the courtesy of Dr. S. Marcus, Univ. of Utah) were used. After passage on slants of hormone blood agar, the organisms were inoculated into 100 ml of Salvin's medium with yeast extract substituted for biotin(5) and grown at 37°C with shaking. Maximal yields of polysaccharide were found after 20 days of growth. At this time, formalin was added to a final concentration of 0.5% and, after 48 hours at 4°C, the cells were separated by centrifugation. The supernate was treated with 40 ml of aqueous saturated sodium acetate solution (pH 7.2) per liter of filtrate and then 2.5 volumes of redistilled 95% ethanol was added. The precipitate of crude polysaccharide was recovered by centrifugation, and an aqueous solution of it was dialyzed against water. After reprecipitation with 2.5 volumes of ethanol, the precipitate was separated by centrifugation, dissolved in distilled water, and deproteinized with a chloroform-n-butanol mixture(6). The sequence of alcohol precipitation and deproteinization was repeated 3 more times, then the final precipitate was dissolved in distilled water, dialyzed thoroughly, and lyophilized.

Column chromatography. DEAE-cellulose‡ was prepared in the phosphate cycle by washing with 0.5 M phosphate buffer (pH 5.5) after first preparing a suspension and treating it cyclically with NaOH and HCl. The suspended adsorbent finally was washed with distilled water until a negative silver nitrate test was obtained. A 1- by 36-inch column was filled by sedimentation with the aqueous suspension of DEAE-cellulose and then packed to a final pressure of 8 psi.§ Other chromatographic data are given in Fig. 1.

Antisera. New Zealand white female rabbits were immunized with living and formalin-killed organisms, given by various routes, with and without adjuvant. Highest titers of antibody (about 1 mg antibody N/ml) were obtained by giving animals 5 weekly intravenous injections of an aqueous suspension (2.2 mg dry weight/ml) of live *H. capsulatum* in 0.85% NaCl. Animals were bled 1 week after the last injection and the serum was filtered through a 0.45-μ Millipore filter, C′ was inactivated by treatment at

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† All steps were carried out at 4°C unless otherwise noted.

‡ Cellex D, Calbiochem, Los Angeles, Calif.

§ Milton Roy Minipump, Milton Roy, Philadelphia, Pa. Monitoring in the ultraviolet region was accomplished using a Gilford model 2000, Gilford Instrument Laboratories, Inc., Oberlin, Ohio.

56°C for 30 minutes, 1% thimerosal (Merthiolate) solution was added to a final concentration of 1:10,000, and the preparation was stored in a frozen state.

Chemical methods. Nitrogen was determined by a modified micro-Kjeldahl method (7), carbohydrate by ferricyanide reduction (8) and by an anthrone method(9) and phosphorus by a molybdate method(10); nucleic acid was calculated from the absorption peak at 260 mμ(11).

Immunologic methods. Quantitative precipitin analyses were carried out by a standard method(12), microimmunoelectrophoresis, as described by Scheidegger(13), and microimmunodiffusion, by the use of Lucite matrices on 25 by 75-mm microscope slides.

Results. Properties of the various preparations are outlined in Table I and Fig. 2. Polysaccharide was not found in the culture medium until 7 days after inoculation and then yields increased to a maximum at 20 days. The weight of organisms isolated reached a maximum in 5 days and remained constant thereafter.

The crude polysaccharide(s) contained large amounts of contaminating protein and nucleic acid (Table I) in spite of repeated treatment with the denaturing mixture of chloroform-n-butanol. Chromatography on DEAE-cellulose in the phosphate cycle led to rapid elution of polysaccharide-containing materials while the substances absorbing at 260 mμ were bound until a gradient in NaCl was started (Fig. 1). All of the chromogen in the starting material was found in fractions VI and VII. The immunologically active fractions were II, IV, and V. These differed in chemical properties from one another and differed from the starting material (Table I) in optical rotation, nitrogen content, and immunologic reactivity. In quantitative precipitin reactions (Fig. 3) and in comparisons of the various fractions by immunodiffusion and immunoelectrophoresis (Fig. 4), fractions II, IV, and V gave strong precipitin reactions but each of these fractions, in turn, probably contained more than one antigen. Fraction II gave a reaction of nonidentity with fractions IV and V; the latter 2 differed in chemical properties. Fractions II, IV, and V also were active (in a 3- to 10-μg dose) in producing delayed hyper-

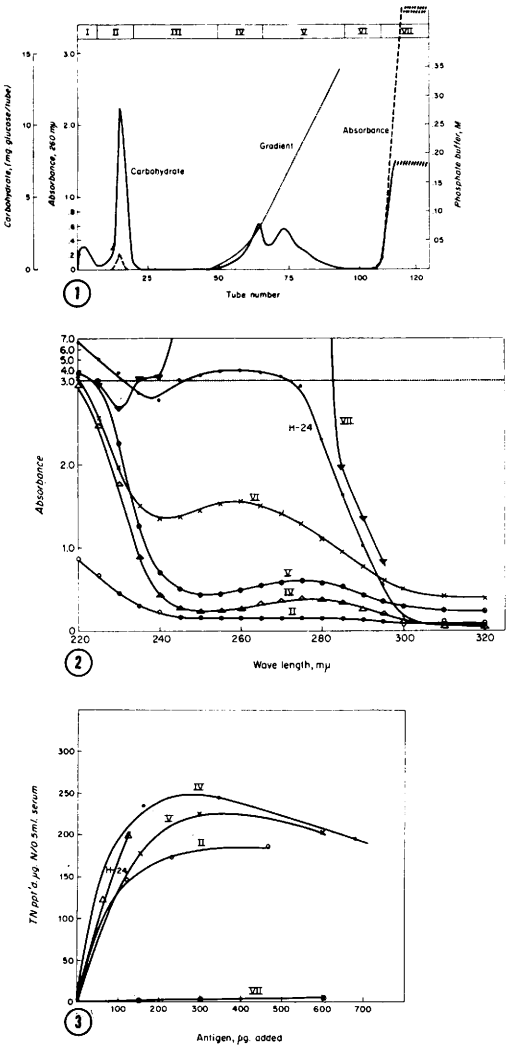

FIG. 1. Elution curve of chromatography of crude *H. capsulatum* polysaccharide on DEAE-cellulose. Fractions, 8 ml per tube; flow rate, 71 ml/hr. Absorbance (dashed line) was read in a flow-through cell at 260 mμ. Gradient in phosphate shown by dotted line; gradient in NaCl (0 to 1 M) started at tube 82 using 0.4 M phosphate buffer (pH 5.5) as the base. Recoveries: starting material (H-24), 358 mg; II, 53 mg; IV, 58 mg; V, 112 mg; VI, 21 mg; VII, 35 mg. Overall recovery, 85% by weight.

FIG. 2. Ultraviolet absorption spectra of crude polysaccharide and chromatographic fractions. Cell length, 1.0 cm; concentration, 1.0 mg/ml. Fractions are identified by Roman numerals corresponding to those in Fig. 1.

FIG. 3. Quantitative precipitin reactions of chromatographic fractions.

TABLE I. Polysaccharide Preparations from *H. capsulatum*.

Prep*	Yields† Cells (g)	Yields† Polysaccharide (g)	$[\alpha]_D$ (degrees)	N (%)	P (%)	Carbohydrate‡ (% as glucose)	RNA (%)
H-25	3.22	.28	+22	3.33	4.8	17.3	11.3
H-26	3.75	.26	+27	3.73	3.4	33.2	9.6
H-27	2.97	.14	+ 8	3.66	3.9	15.4	13.0
H-28	3.15	.61	+57	4.98	2.2	40.5	12.2
H-24	3.24	.23	—	5.57	5.1	23.6	16.6
II	—	—	+69	.85	.2	—	0
IV	—	—	+41	2.47	.2	—	0
V	—	—	+77	2.77	.2	—	0
VII	—	—	—	3.17	2.1	—	—

* H-27 is from strain G-17M; H-28 is from strain 103. The remainder were isolated from strain 6651.

† Per 4.0 l of culture medium.

‡ By an anthrone method(9). Reducing sugar content of the unhydrolyzed preparations by a ferricyanide method(8) was from 5.4 to 8.0%.

sensitivity reactions in sensitized rabbits, with fraction II being somewhat less active than the others.

Discussion. The results presented suggest that *H. capsulatum* elaborates a number of serologically active carbohydrate-containing materials. Immunodiffusion methods indicate that there are at least 2 distinct materials, while the chemical composition of these substances and the results of immunoelectrophoresis show that fractions II, IV, and V each contain at least 2 antigenic components. A marked divergence in yield, optical rotation, and reactivity with anthrone reagent is also evident among the various "strains" of *H. capsulatum* used in this work. Comparisons of these complex mixtures by immunodiffusion also suggests the existence of components of differing serologic reactivity in different "strains." Since such results could be due to chemical alterations during isolation or to simple adsorption of a single antigen on contaminants which differ in composition and electrophoretic mobility, proof of the presence of type-specific components must await isolation of pure materials and direct immunologic comparison. It is also of interest that two distinct components are capable of inducing delayed hypersensitivity reactions in infected animals. However, since both contain nitrogen, the reactivity could be explained on the basis of a single adsorbed protein in all fractions.

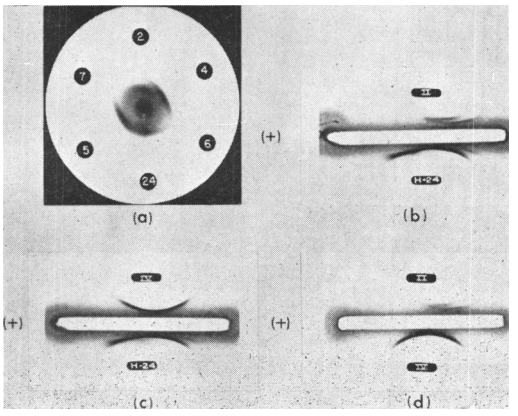

FIG. 4. Immunodiffusion (a) and immunoelectrophoresis (b, c and d) reactions of fractions. Gel diffusion (a) run at pH 7.3 in phosphate buffer for 3 days at 4°C against rabbit anti-*H. capsulatum* serum. Arabic numbers correspond to Roman numerals identifying fractions in Fig. 1 except 24 which is starting material (H-24).

Summary. A mixture of polysaccharides was obtained from culture filtrates of *H. capsulatum* grown in the yeast phase. After fractionation on DEAE-cellulose, 3 chromatographic fractions were obtained which were serologically active. These were free of nucleic acid, contained from 0.85 to 2.77% N and differed both in chemical constants and in immunologic reactivity.

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Relation of Serum Hexosamine to Various Blood Components.* (29011)

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Elevation of serum hexosamines has been reported in a wide variety of clinical conditions(1-6). Several attempts have been made to correlate hexosamine concentrations with other serum components. Shetlar *et al*(3) found an interrelationship between non-glucosamine polysaccharides and glucosamines. West and Clark(1) found no relationship between glucosamine concentrations and serum protein fractions. They did however, observe correlation between high glucosamine content and increased sedimentation rate. Others have noted a relation between protein fractions and blood polysaccharides(7), and between albumin and alpha globulin and glucosamine(8). The present paper presents a comparison of serum hexosamine content with a number of parameters including electrolytes, protein content and leucocyte counts.

Material and methods. Blood samples were obtained from 70 patients at Georgetown University Hospital. History and diagnosis were readily available. Normal blood was obtained from 10 clinically well individuals. Serum hexosamine was determined by Rosenlund's modification of the Elson and Morgan method(9). Glucosamine hydrochloride was used as the standard. Two samples of blood were taken in each case and the hydrolysates of each sample were analyzed in duplicate. The mean of the 4 determinations was used for comparison, the differences among the 4 values was less than 3%. The following blood constituents were determined by the methods indicated, total protein(10), sodium(11), potassium(12), and chlorides (13). Hematocrit, total leucocyte and differential counts were obtained from the hospital laboratory.

Results. Table I gives the hexosamine values in the normals and during various diseases. The normal range is small while the range of values for the pathological conditions is greater. However, the lower values are within the normal range, or even below. For purposes of comparison the blood samples were divided into 2 groups, those with hexosamine values above or below 150 mg per cent. Table II gives the comparison of a number of parameters for these 2 groups. No correlation was found between serum hexosamine content and any of the parameters examined. Comparison of total leucocyte count with hexosamine content, Table III,

TABLE I. Serum Hexosamine Levels.

Diagnosis	No. of patients	Mean values (mg/100 ml)	Range of values
Normal	10	123	117-130
Diabetes mellitus	10	173	128-295
Rheumatism	12	155	122-176
Malignancy	8	156	103-172

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