Purification, Composition, and Serological Characterization of Histoplasmin—H and M Antigens

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Received for publication 16 November 1973

To obtain purified H and M antigens suitable as primary standards in the serological diagnosis of histoplasmosis by agar gel double-diffusion tests, H and M reactive components of histoplasmin were fractionated by column chromatography by using Sephadex G-100, Sephadex G-200, and diethylaminoethyl cellulose. Six fractions from diethylaminoethyl cellulose were reactive in agar gel double-diffusion, complement fixation, and capillary precipitin tests. When examined by electrophoresis on acrylamide gel, one M antigen (fraction 2, molecular weight greater than 200,000) and two H antigens (fractions 5 and 6, molecular weight greater than 200,000) each gave essentially a single protein band. In agar gel double-diffusion and complement fixation tests with sera from patients with proven cases of histoplasmosis, blastomycosis, or coccidioidomycosis, these two fractions of H antigen and the one of M antigen reacted only with sera from proven or suspect cases of histoplasmosis and showed reactivity with those sera known to contain only the anti-H or anti-M antibody, respectively. Fraction 2 (M antigen) and fractions 5 and 6 (H antigens) had carbohydrate-to-protein ratios of 1.60, 0.77 and 0.78, respectively. Both antigens contained galactose, glucose, mannose, and hexosamine, with mannose being the predominant sugar. Fraction 2 was characterized by a high proline and glucose content, whereas fractions 5 and 6 contained higher concentrations of galactose, mannose, glycine, and alanine. Each of these products appeared to separate into two active fractions, one of a molecular weight greater than 200,000 and one of a molecular weight less than 35,000. The M antigen component of fraction 2 was still characterized by a high proline content, whereas the H antigen components of fractions 5 and 6 had a high content of glutamic acid, serine, glycine, and proline.

Presumptive serological diagnosis of histoplasmosis depends, in general, upon two basic analyses: (i) positive complement fixation tests with whole yeast-phase cells or mycelial histoplasmin, and (ii) agar gel double-diffusion tests in which both the H and M precipitin lines are formed (11). A complement fixation titer of at least 1:8 with either antigen is considered evidence of histoplasmosis. Patients with sera demonstrating a precipitin line with the H antigen are usually in the acute and progressive stage of active disease; those demonstrating only the M line are generally in a chronic stage of infection or have had an earlier contact with the causal organisms (10).

Evaluation of patient sera by the agar gel double-diffusion technique depends upon the use of a histoplasmin standard which contains both H and M antigens and a second standard which contains only one of the known antigens. The single antigen standard is necessary to distinguish the precipitation lines formed since it is known that variations in antigen concentration can cause the position of the M and H precipitin lines to be reversed (20). In addition, known standard sera with antibodies to either or both of these antigens are required. Results of this test are much more dependable when reagents are used which contain only a single antigen—H or M. Reagents containing one or the other of these antigens may be prepared by differential fermentation with the correct strain (6, 7, 20) or by separation of the antigens by chemical means. Although preliminary descriptions of the separation of H and M antigens have been reported (4, 8), neither of these antigens has been isolated in a purified condition suitable for use as primary standards and suitable for differential description of their chemical and serological characteristics. Because it would be of benefit to use purified antigens for diagnostic evaluations and because
of our interest in the comparative chemical characteristics of these two antigens, we have attempted to separate and characterize them. Two fractions were obtained which were serologically specific, as H or M antigen, and each appeared to be a single moiety when examined by electrophoresis in acrylamide gel. Upon further investigation, however, each of these separated into two active fractions, one of approximately 35,000 molecular weight and a second of molecular weight greater than 200,000. Certain of the chemical and serological characteristics of these and associated antigens are described.

**MATERIALS AND METHODS**

**Antigen.** Crude histoplasmin (containing 1:10,000 merthiolate) which did not meet the complement fixation titer requirements for use as a standard reagent (18) was supplied by the Microbiological Reagents Unit (W. K. Harrell, Chief).

**Sephadex G-100 chromatography.** Crude histoplasmin was concentrated at least 10-fold on polyethylene glycol 20 (Carbowax) and dialyzed against 0.1 M sodium phosphate buffer, pH 7.0, plus 0.02% sodium azide. A sample containing 75 to 150 mg of protein was eluted from a column (2.5 by 100 cm) by ascending chromatography with the dialysis buffer. Fractions containing 200 mg of protein were collected at a flow rate of 8 to 10 drops per min and were tested for activity by the capillary precipitin test. Active fractions were pooled and concentrated.

**Ion exchange chromatography.** The pooled fractions from Sephadex G-100 were dialyzed against 0.1 M tris(hydroxymethyl)aminomethane (Tris) buffer, pH 8.0. A sample containing 65 mg of protein was applied to a column (1.2 by 11 cm) of Whatman diethylaminoethyl (DEAE) cellulose DE 52 which had been equilibrated with the dialysis buffer. The sample was eluted with 0.1 M Tris buffers of descending pH (8.0, 7.0, 6.0, 5.0, 4.0, and 4.0 plus 0.1 M NaCl). Fractions (2.5 ml) were tested for activity by capillary precipitin, agar gel double-diffusion, and complement fixation tests. They were pooled according to the content of H or M antigen as shown by agar gel double diffusion.

**Electrophoresis.** An Ortec 4200 electrophoresis system was used along with a 4100 Pulsed constant power supply (Ortec, Inc., Oak Ridge, Tenn.). A sample containing 200 mg of protein was dried in a vacuum desiccator over concentrated sulfuric acid, and was electrophoresed according to the procedure outlined in the instruction manual for casting 4.5, 6, 8% gradient gel. Gels were stained with amido black and scanned with a Canalo model G microdensitometer at 570 nm equipped with a model 8G recorder (Canalo, Inc., Rockville, Md.). Agarose gel immunoelectrophoresis was conducted by using 0.0375 M barbital-sodium barbital buffer, pH 8.5, at 250 V for 1 h with an approximately 8 mA current. The lines of precipitate were formed by using human M and H plus M-specific human antiserum; the slides were stained with amido black. These analyses were done by Herman Gross.

**Paper chromatography.** Samples for amino acid analysis containing 500 µg of protein were made 6 N with HCl, sealed under vacuum, and heated for 18 h in a boiling-water bath. The samples were dried in a vacuum desiccator containing concentrated sulfuric acid and a dish of sodium hydroxide pellets, and were dissolved in water. The drying process was repeated three times. Chromatographic solvents and developing sprays were those of Cummins and Harris (3). For sugar analysis, samples containing 500 µg of carbohydrate were made 2 N with sulfuric acid, flushed with nitrogen, sealed, and heated 2 h in a boiling-water bath. Hydrolyzed samples were neutralized with a saturated solution of barium hydroxide, centrifuged, lyophilized, and re dissolved in water. Chromatographic solvents and sprays were those of Cummins and Harris (3).

**Chemical analysis.** Each DEAE cellulose fraction was analyzed for reducing sugars by the method of Park and Johnson (14); total carbohydrate was determined by the phenol-sulfuric acid method of Dubois et al. (5); protein was determined by the method of Lowry et al. (12); and hexosamine was determined by a modified Elson-Morgan method (1). Glucose and galactose were determined enzymatically by Glucostat and Galactostat reagents, respectively (Worthington Biochemical Corp., Freehold, N.J.). Amino acid analyses (12) were performed by the Biochemistry Unit, Center for Disease Control, C. Wayne Moss, Chief. Quantitative measurements were made according to the procedure of Gehrke et al. (9) with 2,4-diaminobutyric acid as an internal standard.

**Serology.** Quantitative capillary precipitin tests were performed with CDC serum 5735, which contains both H and M antibodies (15). For agar gel double diffusion (2), serum 61, which contains only M antibodies, was used in addition to serum 5735. The agar medium, developed by Paul Nichols, was modified to contain 2.25 g of sodium chloride, 0.50 g of dibasic potassium phosphate, 2.5 g of Noble agar, 0.50 ml of 10% aqueous magnesium chloride, 1.0 g of sodium citrate, and 0.05 g of sodium azide in 250 ml of water. The Laboratory Branch complement fixation test was performed on all DEAE cellulose fractions (18). Specificity of the antigens in fractions 2 and 5 was tested by the Fungus Immunology Section, Center for Disease Control, Leo Kaufman, Chief.

**Molecular weight determinations.** A sample (3.0 mg of protein) was dried at 5°C over concentrated sulfuric acid and dissolved in 0.10 M sodium phosphate buffer at pH 7.0. The sample was applied to a column (1.8 by 30.7 cm) of Sephadex G-200 and eluted with the same buffer. Calculations were made by the equations of Squire (17).

**RESULTS**

**Preliminary observations.** With few exceptions, histoplasmin used in these studies was prepared by the Biological Reagents Section but, due to low complement fixation titer or absence of H and M antigens, did not meet the performance specifications for a biological reagent distributed for reference use by the Scien
tific Resources Branch, Center for Disease Control. Various combined lots generally had low concentrations or no apparent precipitin antigens. Thus the primary step was concentration, and several procedures were tested by using high-titered reagent histoplasmin. These procedures included alcohol or acetone precipitation, vacuum distillation, dialysis with Carbowax, and precipitation at pH 3.0 with or without MgCl₂ at concentrations of 1% or less. Certain of these results and those of associated experiments are described briefly. Precipitation with 60 to 80% alcohol (at pH 7.5) gave complete recovery of both H and M antigen, whereas precipitation at pH 3.0 with or without 60% alcohol resulted in either partial or complete loss of M antigen. Precipitation with 75% acetone (at pH 7.5) gave 50% or greater inactivation of both antigens. Addition of MgCl₂ before acetone was added did not increase recoveries, although precipitation was increased. Vacuum distillation at temperatures no greater than 30°C permitted 100% recoveries of both antigens, but the procedure was time consuming and no purification was effected. Alcohol precipitation greatly reduced the amount of contaminating components observed during Sephadex column fractionation; at the levels of histoplasmin concentration tested, these substances were equally well removed during the simple Sephadex purification. The concentration procedure selected for use was dialysis concentration in a 1.87-inch (about 4.7 cm) diameter tubing placed in a Carbowax bath with mild, constant agitation on a shaking machine at 5°C. Concentrated products contained considerable dark brown to blue-black color. Decolorization of the antigens was attempted with six absorbants. Some absorbants had little effect upon the color of the solution or the activity of the antigens (Celite 547, 545, or 535), whereas others completely decolorized the solutions but totally inactivated the antigens (coconut activated charcoal, Norite A, decolorizing charcoal-acid). Because of disadvantages observed with absorbants, none were used and the final products obtained (see Results) had a faintly discernible color.

**Chromatography on Sephadex G-100 and G-200 columns.** In general, the results obtained with various lots of histoplasmin were the same. After concentration by Carbowax, two major peaks were observed with Sephadex G-100. Precipitin and complement fixation activities were restricted to the first peak appearing in the fraction eluted at the void volume and extending to fraction 60 (Fig. 1). Combination of these active fractions or initial fractionation on Sephadex G-200, or both, gave three separate fractions (Fig. 2), with the activity being limited, as determined by agar gel double-diffusion or capillary precipitin tests, to those fractions appearing at the void volume and extending through fraction 35 (Fig. 2). When fractions 28 to 40 (Fig. 2) eluted in phosphate buffer were dried over sulfuric acid and reapplied to the column, essentially the same elution profile was obtained as observed originally. Although there was a slight decrease in the peak observed from fractions 45 to 60, a peak was again observed between fractions 65 and 100. Similar results were obtained with combined fractions 45 to 60.

![Fig. 1. Ascending chromatography of crude 10× concentrated histoplasmin on a column (2.5 by 100 cm) of Sephadex G-100. The sample was eluted with 0.1 M phosphate buffer, pH 7.0, at a flow rate of 8 to 10 drops per min. Precipitin activity per milliliter of eluate (2.5-ml fractions) is indicated by bar graphs; optical density was read at 254 nm.](image-url)
although a great reduction in the peak at the void volume was observed.

Removal of low-molecular-weight inactive material increased the specific activity in the complement fixation test and left the carbohydrate-to-protein ratio essentially unchanged. Depending upon the lot of histoplasmin tested, the carbohydrate-to-protein ratio of the combined fractions ranged from 2.0 to 1.0. Subsequently, larger columns of Sephadex G-100 were used, and the general characteristics and recoveries of fractions were not altered. All fractions showing capillary precipitin reactivity were combined for further purification on DEAE cellulose.

DEAE cellulose fractionation. Ion exchange chromatography with DEAE cellulose separated the Sephadex G-100 purified histoplasmin into seven serologically active fractions (Fig. 3). Although the first fraction eluted (0-15) had capillary precipitin reactivity, it had no complement fixation titer and showed no precipitin bands in agar gel double diffusion. The other six fractions were positive in all three tests (Table 1). As the pH of the eluting buffer decreased, the fractions showed a rough decrease in carbohydrate-to-protein ratio from 1.15 to 0.42; the fractions had 280/260 absorption ratios ranging from 1.22 to 1.02, which suggested a nucleic acid concentration no greater than 2 to 3%. Examination of the fractions by continuous absorption spectrum from 220 to 660 nm gave a smooth curve with a single peak at 276 nm for fractions 2 and 5. The remaining fractions had a single maximum between 275 and 280. Nevertheless, at concentrations of 1.0 mg/ml, the solutions of the fractions had a brown color for which there was no specific absorption peak. Preliminary examination of the fractions by agar gel double diffusion (Fig. 4) indicated that fraction 1 had no H or M antigen, fractions 3 and 4 had H and M antigens in different proportions, fraction 2 contained only M antigen (Fig. 4 and 5), and fractions 5, 6, and 7 had only H antigen (Fig. 4, fraction 7 not shown).

Evaluation of the specific complement-fixing activity (reciprocal of complement-fixing titer, milligrams of protein) showed a wide disparity between fractions 2 to 7; this disparity was associated with the relative presence of M and H antigens of the preparations (Table 1). Thus, fractions 2 and 3 showed approximate specific activities of 600 and 250, respectively, whereas fractions 5 and 6 each had approximate specific activities of 25. Without question, this is a result of the relative concentration of specific antibodies present in the serum (CDC human antihistoplasmosis serum, lot 29) used for the complement fixation test, but it may indicate a relative inherent difference in the two antigens in regard to reactivity. The serum was only known to have both H and M antibodies. On the
TABLE 1. Characteristics of fractions separated by DEAE cellulose chromatography

<table>
<thead>
<tr>
<th>Fraction no.</th>
<th>Tubes collecteda</th>
<th>Elution pH</th>
<th>A₂₈₀ max</th>
<th>CHO/protein</th>
<th>Maximal absorption (nm)</th>
<th>Capillary precipitin</th>
<th>No. of protein bandsb</th>
<th>Complement fixationc</th>
<th>Agar gel Double diffusion</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1-10</td>
<td>8.0</td>
<td>1.082</td>
<td></td>
<td>276</td>
<td>+4</td>
<td>1</td>
<td>Neg.</td>
<td>H M</td>
</tr>
<tr>
<td>2</td>
<td>17-20</td>
<td>7.4-7.2</td>
<td>1.159</td>
<td></td>
<td>275-280</td>
<td>+5</td>
<td>1</td>
<td>598</td>
<td>+ +</td>
</tr>
<tr>
<td>3</td>
<td>25-35</td>
<td>7.2-6.6</td>
<td>0.798</td>
<td></td>
<td>275-280</td>
<td>+2</td>
<td>2</td>
<td>248</td>
<td>+ +</td>
</tr>
<tr>
<td>4</td>
<td>65-75</td>
<td>6.3-5.9</td>
<td>0.527</td>
<td></td>
<td>275-280</td>
<td>+3</td>
<td>2</td>
<td>49</td>
<td>+ +</td>
</tr>
<tr>
<td>5</td>
<td>110-120</td>
<td>5.6-5.2</td>
<td>0.768</td>
<td></td>
<td>276</td>
<td>+2</td>
<td>2</td>
<td>28</td>
<td>+ ±</td>
</tr>
<tr>
<td>6</td>
<td>140-143</td>
<td>4.8-4.5</td>
<td>0.779</td>
<td></td>
<td>275-280</td>
<td>+1</td>
<td>1</td>
<td>22</td>
<td>+ ±</td>
</tr>
<tr>
<td>7</td>
<td>170-172</td>
<td>4.5-1.5</td>
<td>0.417</td>
<td></td>
<td>275-280</td>
<td>+1</td>
<td>&gt;3d</td>
<td>5</td>
<td>± ±</td>
</tr>
</tbody>
</table>

*a Fractions 1 through 7 contained 9, 9, 13, 18, 28, 7, and 5%, respectively, of the total protein placed on the column.

*b Number of bands detected by microdensitometer after electrophoresis of samples on polyacrylamide gel.

*c Complement fixation results are expressed as the end-point dilution factor divided by the amount (in milligrams) of protein. The complement fixation value of the Sephadex G-100 concentrate originally placed on the DEAE column was 410.

*d These peaks were not much higher than the background. There were, however, at least three peaks.

Fig. 4. Agar gel double diffusion of DEAE cellulose fractions versus serum 5735 (anti-H and -M).

The fractions were examined further by agarose immunoelectrophoresis or acrylamide gel electrophoresis, or both. Immunoelectrophoresis patterns showed only two protein bands; the H antigen migrated more rapidly. Examination of the stained acrylamide gel obtained with 200 µg of each fraction showed only two protein peaks in fractions 2 to 6; fraction 7 contained multiple bands from the origin to the bottom of the gel of which none were discrete. The presence of H antigen was observed in fraction 7 only by agar gel double diffusion—no discrete peak was observed at the H antigen locus of the acrylamide gel. Examination of the acrylamide gel electrophoresis patterns (Fig. 6 and 7) showed a slight asymmetry of the M peak of fraction 2, which suggested as much as a 12% contamination with other products. However, the complete absence of H antigen in fraction 2 was verified by extensive agar gel double-diffusion tests. Similar examination of fraction 5 (Fig. 7) showed a minor contamination with M antigen and an asymmetry of the H peak in fraction 6 which indicated only 91% purity of the protein of this fraction. M antigen was absent from fraction 6. Direct visual examina-

Fig. 5. DEAE cellulose fractions 2 and 5 versus sera 5735 (anti-H and -M) and 61 (anti-M) in agar gel double diffusion.
HISTOPLASMIN H AND M ANTIGENS

Analyses were analyzed qualitatively for sugars by paper chromatography. Four spots identified by standards as glucose, galactose, mannose, and a hexosamine were found. From the chromatograms it appeared that mannose was the main sugar component in each fraction. Quantitative analysis showed that, generally, the concentration of glucose decreased from fraction 1 to fraction 7, whereas that of galactose increased (Table 2). Thus, fraction 2, the purest M antigen, contained twice as much glucose and about one-third as much galactose and mannose as did fractions 5 and 6, the purest H antigens. Paper chromatography indicated a more or less complete spectrum of amino acids present in each DEAE cellulose fraction, but did not distinguish between the different fractions. Quantitative analysis, however, showed major differences between the relative amino acid

![Fig. 6. Microdensitometer scans of M antigen fractions on polyacrylamide gels after electrophoresis of 200 μg of DEAE cellulose fractions. Samples were stained with amido black, destained with 10% acetic acid, and scanned at 570 nm.](image)

![Fig. 7. Microdensitometer scans of polyacrylamide gels after electrophoresis of DEAE fractions 5 and 6. See legend for Fig. 6.](image)

### TABLE 2. Relative concentration of sugars in fractions from DEAE cellulose chromatography (μmol)

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Glucose</th>
<th>Galactose</th>
<th>Mannose</th>
<th>Hexosamine</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3.20</td>
<td>1.0</td>
<td>7.0</td>
<td>1.2</td>
</tr>
<tr>
<td>2</td>
<td>2.10</td>
<td>2.5</td>
<td>5.7</td>
<td>1.0</td>
</tr>
<tr>
<td>3</td>
<td>3.60</td>
<td>3.3</td>
<td>15.4</td>
<td>1.0</td>
</tr>
<tr>
<td>4</td>
<td>1.00</td>
<td>3.9</td>
<td>5.0</td>
<td>1.0</td>
</tr>
<tr>
<td>5</td>
<td>1.00</td>
<td>7.7</td>
<td>13.6</td>
<td>1.5</td>
</tr>
<tr>
<td>6</td>
<td>1.00</td>
<td>7.3</td>
<td>14.9</td>
<td>2.7</td>
</tr>
<tr>
<td>7</td>
<td>1.00</td>
<td>2.7</td>
<td>5.9</td>
<td>1.1</td>
</tr>
</tbody>
</table>
concentration of the different fractions (Table 3). The concentration of arginine, isoleucine, and phenylalanine was low in all fractions, whereas that of aspartic acid was generally high. Fractions 5 and 6, the purest H antigens, had higher concentrations of alanine and glycine and lower concentrations of threonine, serine, and proline than fraction 2, the purest M antigen. There were differences between the two H fractions; fraction 6 had about twice as much proline, aspartic acid, tyrosine, and lysine as fraction 5. Fraction 2 was distinguished by a high proline concentration (Fig. 8), whereas fractions 3 and 4 had very high aspartic acid content. Thus the proteins present in fractions 2, 3, and 4, and 5 and 6 differ significantly. This may be due to differences in associated proteins or differences in composition of the antigens themselves.

**Serological activity of purified fractions.**

The purified H (fractions 5 and 6) and M (fraction 2) antigens were tested against human sera from patients with proven or suspected cases of histoplasmosis, blastomycosis, and coccidioidomycosis. Agar gel double-diffusion testing of fractions 2 and 5 revealed no H present in the M antigen and vice versa with the 26 histoplasmosis sera used. Eight of these sera were from proven cases, and the others had been tested against CDC standard antigens lots 8 and 9.

Tests for cross-reactivity showed no precipitin bands were formed with fractions 2 or 5 with any of the eight sera from proven cases of coccidioidomycosis. There was a positive reaction between the H antigen and one of the eight blastomycosis sera. Fraction 6 was tested against 20 histoplasmosis sera, 5 blastomycosis sera, and 5 coccidioidomycosis sera. It showed no cross-reactivity with the coccidioidomycosis or blastomycosis sera. No M activity could be demonstrated in fraction 6.

**TABLE 3. Comparison of relative concentration of amino acids of fractions separated by DEAE cellulose chromatography**

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alanine</td>
<td>2.553</td>
<td>2.552</td>
<td>2.031</td>
<td>4.514</td>
<td>3.828</td>
<td>4.256</td>
<td>1.702</td>
</tr>
<tr>
<td>Glycine</td>
<td>2.710</td>
<td>3.608</td>
<td>2.072</td>
<td>2.764</td>
<td>5.415</td>
<td>5.417</td>
<td>3.609</td>
</tr>
<tr>
<td>Serine</td>
<td>1.976</td>
<td>6.209</td>
<td>1.752</td>
<td>1.558</td>
<td>2.921</td>
<td>2.393</td>
<td>2.590</td>
</tr>
<tr>
<td>Valine</td>
<td>1.168</td>
<td>3.113</td>
<td>2.072</td>
<td>1.106</td>
<td>2.487</td>
<td>1.659</td>
<td>1.106</td>
</tr>
<tr>
<td>Leucine</td>
<td>1.244</td>
<td>1.104</td>
<td>0.762</td>
<td>1.265</td>
<td>0.562</td>
<td>0.652</td>
<td>0.562</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>0.846</td>
<td>0.561</td>
<td>0.423</td>
<td>3.460</td>
<td>3.705</td>
<td>7.908</td>
<td>5.435</td>
</tr>
<tr>
<td>Proline</td>
<td>2.965</td>
<td>11.847</td>
<td>5.578</td>
<td>3.460</td>
<td>3.705</td>
<td>7.908</td>
<td>5.435</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>2.479</td>
<td>5.660</td>
<td>19.123</td>
<td>15.112</td>
<td>3.540</td>
<td>6.611</td>
<td>3.305</td>
</tr>
<tr>
<td>Methionine</td>
<td>1.141</td>
<td>1.013</td>
<td>1.141</td>
<td>1.015</td>
<td>0.761</td>
<td>1.014</td>
<td>0.970</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>1.000</td>
<td>1.000</td>
<td>1.000</td>
<td>1.000</td>
<td>1.000</td>
<td>1.000</td>
<td>1.000</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>2.209</td>
<td>6.351</td>
<td>4.731</td>
<td>5.047</td>
<td>3.154</td>
<td>5.880</td>
<td>3.784</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>1.552</td>
<td>3.099</td>
<td>2.069</td>
<td>1.725</td>
<td>1.293</td>
<td>3.450</td>
<td>1.379</td>
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<tr>
<td>Lysine</td>
<td>1.093</td>
<td>1.457</td>
<td>1.367</td>
<td>1.458</td>
<td>1.093</td>
<td>3.646</td>
<td>1.457</td>
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<tr>
<td>Arginine</td>
<td>0.735</td>
<td>0.326</td>
<td>0.734</td>
<td>0.653</td>
<td>0.245</td>
<td>0.326</td>
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</table>

*Relative amino acid concentration (millimoles) calculated on the basis of phenylalanine with a mole concentration of 1.*
Separation of purified H and M antigens into smaller units. When fractions 2, 5, and 6 were concentrated and applied to a Sephadex G-200 column for molecular weight determination, each antigen yielded three to four peaks (Fig. 9). Peak I of the M antigen, molecular weight greater than 200,000, had M serological activity only; peak II, molecular weight 25,000, showed an M band plus an unknown band in agar gel double diffusion; two remaining peaks, III and IV, were inactive. The elution pattern of the H antigen was similar to that of the M antigen. H specificity was found in the fraction of molecular weight greater than 200,000; a second fraction, molecular weight 30,000, gave an H band plus another band in agar gel, and two inactive fractions of lower molecular weight (Fig. 9).

Further chemical and serological analyses of the separation products were limited because of the small amount of antigen that remained. Analysis by paper chromatography showed that fractions I and II of both antigens contained the same sugar components as the original DEAE cellulose fractions. Quantitative sugar analysis showed a change in the glucose-to-galactose ratio of the high molecular weight fractions; H fraction I and M fraction I had ratios of 4.2 and 4.8, respectively.

Amino acid analysis showed similarities between the low-molecular-weight fractions. The concentrations of arginine, methionine, leucine, alanine, tyrosine, and glycine were equal; there was a small difference between the concentrations of isoleucine, phenylalanine, lysine, and aspartic acid, and the concentration of proline in M fraction II was twice as high as that in H fraction II (Fig. 10).

In the high-molecular-weight fractions there were equal concentrations of isoleucine, phenylalanine, and leucine, with greater differences between the remaining amino acids (Fig. 11). Only a trace of tyrosine remained in the high-molecular-weight fractions, and arginine and methionine were not detected. The overall difference between fractions MI and HI is much

![Fig. 9. Spontaneous separation of purified H and M antigens into smaller units. Purified M antigen (DEAE cellulose fraction 2) and purified H antigen (DEAE cellulose fraction 6) were applied to a column (1.8 by 30.7 cm) of Sephadex G-200. A sample containing 3 mg of protein was eluted with 0.1 M sodium phosphate buffer at pH 7.0. Forty-drop fractions were collected at a flow rate of 8 to 10 drops per min and pooled. Lower graph: fraction 2I = tubes 18 to 23; fraction 2II = 24 to 39; fraction 2III = 40 to 65; fraction 2IV = 65 to 90. Top graph: fraction 5I = 18 to 25; 5II = 26 to 50; 5III = 51 to 70; 5IV = 71 to 90.](http://iai.asm.org/)
the high-molecular-weight fractions less than that of the original DEAE cellulose fractions 2 and 5. When the fractions were analyzed for proteins, 20 to 24% was found in the high-molecular-weight fractions, 59 to 63% was in the low-molecular-weight fractions, and 13 to 20% in the two inactive fractions.

**DISCUSSION**

The ultimate purpose of this investigation was to obtain purified and stable H and M antigen preparations which may be used as primary standards for serological diagnosis and as immunogens to prepare specific antisera. At the same time, it was of interest to determine the relative chemical characteristics of both antigens. These determinations are of value from the standpoint of understanding the immunological aspects of the products and ultimately pinpointing the serologically reactive site of the purified antigen.

The results of experiments reported here show that the H and M antigens of histoplasmin are formed as a complex of at least five separate H antigens and three M antigens. Chromatography and electrophoretic data suggest that the H and M antigens each may be readily separated from one another using a combination of DEAE chromatography and electrophoretic mobility.

However, for the present, only three fractions (one M antigen and two H antigens) have been obtained which showed serological specificity. Although several experiments have shown that the individual M and H antigens may occur as a protein-carbohydrate complex which may migrate as a single major protein entity electrophoretically, the results obtained in regard to color, products of DEAE fractionation, and protein contamination observed in acrylamide gel electrophoresis, show that DEAE fractions 2, 5, and 6, although serologically specific, are by no means pure.

The results obtained with Sephadex 200 and DEAE column chromatography showed that these antigens have a wide range of molecular weights, but we suggest that they show a tendency to form high-molecular-weight complexes in the histoplasmin concentrate. Consequently, passage of such concentrates through combined Sephadex and DEAE columns may yield fractions which still demonstrate low-molecular-weight products believed removed by fractionation with Sephadex G-100. Thus, histoplasmin concentrates passed through a 50,000 molecular weight sieve (Amicon) showed no H or M antigen activity. However, fractions in phosphate buffer taken at or near the void volume of Sephadex G-200 columns showed molecular weight components of 30,000 or less.
after they were brought to dryness and again passed through Sephadex G-200. Similarly, when the combined active fractions of Sephadex G-100 were passed through DEAE cellulose, three of the single protein eluates obtained at a given pH showed a three-peak elution pattern in Sephadex G-200 which was similar to, if not the same as, that observed with the original histoplasmin concentrate. However, we recognize that these interpretations are limited in part due to the overlap of various proteins taken from fractions of closely proximal chromatographic peaks.

The H and M antigens of histoplasmin are most probably the products of autolytic breakdown of the mycelial mat rather than of the direct formation and excretion of a growth product. The concept of antigen release due to autolytic decomposition of the mycelial mat is supported by the work of Ehrhard and Pine (7) and Schubert and Wiggins (16). The high carbohydrate-to-protein ratio observed in these preparations suggests that the antigens emanate from cell wall decomposition products. Unpublished results (Pine and Bradley) of experiments in which Sephadex G-100-purified histoplasmin and DEAE cellulose-purified H and M antigens were treated with lysozyme, chitinase, trypsin, or chymotrypsin failed to implicate either the carbohydrate or the protein portion as the site of antigenic reactivity. Although the purified antigens were inactivated by heating 10 min in a boiling-water bath, none of the enzymatic treatments caused a loss of reactivity in the agar gel double-diffusion tests. The results of DEAE cellulose fractionation suggest that multiple breakdown products are released from the same source. Each of the multiple M fractions and H fractions released differs from the other members of its respective H or M antigenic group by its isoelectric point. In addition, the overall chemical compositions of the H and M antigens are similar, although the M antigens are distinguished from the H antigens by very much higher concentrations of proline and higher concentrations of aspartic acid, serine, threonine, glutamic acid, and glucose. The much closer relationship of M to H antigen was made more obvious by the analysis of the separation products of purified fraction 2 (M antigen) and fraction 5 (H antigen). Both products gave rise to two active fractions, one of a molecular weight greater than 200,000 and one of a molecular weight of approximately 35,000. Although each high-molecular-weight separation product retained its antigenic specificity, the two products appeared quite similar in amino acid composition (Fig. 10), whereas it was the two low-molecular-weight fractions which continued to exhibit the differential chemical compositions initially observed between fractions 2 and 5. Furthermore, agar gel double-diffusion tests showed that these low-molecular-weight fractions were not pure, since additional agar gel bands were observed in both products by use of adequate serum. Immunization of rabbits with the DEAE cellulose purified H fraction (fraction 5) gave sera which were highly reactive in capillary precipitin tests and which showed five to eight different precipitin lines but no H or M lines by the agar gel double-diffusion test. Thus, the overall results suggest that both the H and M antigens which we have obtained contain multiple immunogenic sites which may reside on the same molecule or on different molecules closely associated either by covalent or electrostatic bonds.

During the past several years, three different preparations of purified fractions of H (DEAE cellulose, fraction 5) and two preparations of purified fraction of M (DEAE cellulose fraction 2) were evaluated by the Mycology Unit for specificity with diverse sera. Each of these products was specific for H or M; no cross-reactions were observed. They showed excellent reactivity with diverse histoplasmosis case sera. Nevertheless, the results obtained (with the low-molecular-weight antigens) suggest that products which contain more desirable features of stability and reactivity can be found. It would appear from preliminary trials that as immunogens these antigens require further purification or elimination of cross-reacting sites.

ACKNOWLEDGMENTS

We thank Leo Kaufman and David McLaughlin for their serological evaluation of several purified H and M antigens.

LITERATURE CITED


