Hemagglutinating Property of *Haemophilus aegyptius*

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Extracts possessing the capacity to hemagglutinate normal human erythrocytes were recovered from *Haemophilus aegyptius* by treatment with either diethylene glycol or acetone. Antisera prepared against these extracts or the unextracted bacterial cell inhibited hemagglutination by homologous and heterologous antigens. Microgel diffusions indicated the presence of identical components in each extract as expressed by lines of identity between antisera to each fraction. The hemagglutinin was identified as a lipopolysaccharide, 42% lipid and 57% carbohydrate. The determination of 6% phosphorus in the lipid fraction identified it as containing phospholipid.

In 1949, during an investigation of epidemic acute conjunctivitis in the lower Rio Grande Valley of Texas, Pittman and Davis (12) found that a member of the genus *Haemophilus* was the apparent cause of the infection. Although this organism resembled *H. influenzae*, its properties were more characteristic of the Koch-Weeks bacillus isolated from individuals suffering from a benign conjunctivitis (7). In 1949, Davis, Pittman, and Griffiths (1) reported that strains of *H. aegyptius* possessed the capacity to hemagglutinate human red blood cells, thereby differing from the nonencapsulated, nontype-specific *H. influenzae* strains. The hemagglutinating capacity of *H. aegyptius* was shown to be inhibited by specific antiserum prepared in rabbits. This property could not be completely removed from the organism by successive washings with normal saline. From this time until the recent investigations by Rheins et al. (13), little has been published regarding this distinctive serological property. These workers developed a method for the extraction of large amounts of serologically active hemagglutinin by treatment of cell masses with alkaline saline (pH 9.5 to 10.0). Upon acidification to pH 6.5, a precipitate was recovered which would hemagglutinate human erythrocytes. At a higher pH (8.5), this material would sensitize red cells but would not directly hemagglutinate. Preliminary studies regarding the chemical identity of this substance indicated a predominance of polysaccharide. The purpose of this investigation was to characterize this property further.

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**Materials and Methods**

*H. aegyptius* strains were obtained from R. E. Weaver (Communicable Disease Center, Atlanta, Ga.) and Margaret E. Pittman (National Institutes of Health, Bethesda, Md.). The strains supplied by Dr. Weaver were isolated from Yaqui Indians in Arizona during an outbreak of acute conjunctivitis. Cultures were routinely passed and maintained on Brain Heart Infusion medium fortified with 5% peptic digest of sheep blood (which supplies the necessary X and V factors) after the method of Fildes (3).

Large quantities of *H. aegyptius* cells were obtained by cultivating the various strains in 32-oz (0.946-liter) prescription bottles containing the fortified medium. Organisms were harvested after 18 to 24 hr of incubation by washing the cells from the agar surface with unbuffered physiological saline. The resulting suspensions were washed three times in normal saline and tested for the capacity to directly hemagglutinate human erythrocytes.

Modifications of the technique employed by Morgan (9) to obtain specific antigenic substances from *Shigella dysenteriae* were applied to obtain hemagglutinin from *H. aegyptius* cells. The packed washed cells from one prescription bottle were suspended in 10.0 ml of diethylene glycol. Pools from six bottles (totaling 60 ml) were added to a 125-ml Erlenmeyer flask and shaken overnight at 4°C. Subsequently, the mixture was divided into tubes and centrifuged at 12,000 rev/min in a Sorvall Superspeed centrifuge for 5 min. The supernatant fluid was then filtered through double prefilters (Millipore Corp., Bedford, Mass.) to ensure the removal of bacteria. Addition of the diethylene glycol extract to dialysis tubing was followed by dialysis against distilled water at room temperature until the alcohol was no longer apparent in the sample. The water suspension of this extract was concentrated by pervaporation and stored.
by freezing in vaccine bottles until chemical and serological tests were performed.

In a similar manner, washed and packed \textit{H. aegyptius} cells were suspended in acetone. The volumes and methods used were the same as those described above for extraction by diethylene glycol.

Preparations of the various strains of \textit{H. aegyptius} for the immunization of rabbits were made from 18- to 24-hr cultures. The washed, packed cells were suspended in physiological saline containing 1:10,000 merthiolate as a preservative; the suspensions were standardized to a turbidity of 10 billion cells per ml. This was accomplished by dilution of the suspension against an appropriate turbidity standard in a Coleman Junior spectrophotometer. These were then introduced into vaccine bottles and heat-inactivated in a water bath at 56 C for 30 min.

In an attempt to obtain antibodies directed specifically against the hemagglutinin without interference from extraneous material from either bacterial cell or medium, normal human red cells were modified with acetone extract and injected as antigens. To accomplish this, one volume of washed, packed human erythrocytes was incubated with two volumes of the acetone-extracted hemagglutinin made isotonic by admixture of salt. After incubation at 37 C for 30 min, the modified red cells were washed three times in saline to remove unadsorbed material and were reconstituted with saline to twice the original volume of the packed cells. Erythrocytes prepared in this manner were tested for passive hemagglutination before injection into rabbits.

Isotonic hemagglutinin recovered by acetone extraction was also used independently as antigen in an attempt to demonstrate antibody production without the use of a carrier substance.

Antisera against the various strains of intact \textit{H. aegyptius} cells and extract-modified red cells and extract alone were prepared by administering intravenously, into young adult rabbits, 1.0-ml quantities of the desired antigen at 3-day intervals for a 9-day period. On the 15th day after the first injection, each animal was bled by cardiac puncture and the resulting sera were stored at --4 C until needed.

Extracts prepared by treatment of \textit{H. aegyptius} cells with diethylene glycol and acetone were tested for hemagglutinability. Where needed, the extracts were made isotonic and serial twofold dilutions were prepared in saline through the titration series. To each tube, 0.5 ml of a 0.5% suspension of normal human erythrocytes was added. All tubes were shaken, incubated at 37 C for 30 min in a water bath, and allowed to settle at room temperature. The titration end point was considered the highest dilution exhibiting a definite pattern of hemagglutination.

To verify that the capacity to hemagglutinate human red cells was a property of the \textit{H. aegyptius} species and not an extraction artifact, \textit{H. influenzae} cells (nonencapsulated) were also extracted with acetone. The final concentration was tested for hemagglutinability by employing serial doubling dilutions as described above.

Antisera prepared against red cells modified with acetone-extracted hemagglutinin, intact bacterial cells, and isotonic acetone extract were tested for the capacity to inhibit the hemagglutinating activity of each original antigenic preparation after absorption with normal human erythrocytes. A predetermined amount (4 units) of the hemagglutinating agent was added to 0.25 ml of each serial dilution of antiserum. After incubation at 37 C for 30 min, 0.5 ml of a 0.5% suspension of normal human red cells was added to each tube; the test series was reincubated for 30 min at 37 C. Upon removal and completed sedimentation at room temperature, all tubes were examined for hemagglutination inhibition. The last dilution exhibiting inhibition of hemagglutination was considered the end point of the titration. In an attempt to demonstrate whether the various extracts differed in their antigenic composition, the antisera described above were diluted 1:10 and cross-adsorbed with the variously extracted hemagglutinins. Hemagglutination-inhibition tests were thus repeated with homologous and heterologous systems.

To further demonstrate the similarity of hemagglutinin extracted by the above methods, antisera from each preparation were diffused against the soluble alkaline-extracted hemagglutinin obtained by Rheins (13) by the microgel technique of Yakulis and Heller (15). The gel consisted of 1% Noble agar in glycine buffer (pH 8.6) pipetted onto microscope slides. After incubation of the slides in moisture chambers at 37 C for 24 hr, the preparations were examined for lines of precipitation.

The alkaline-extracted hemagglutinin obtained earlier by our investigation of \textit{H. aegyptius} was shown to contain appreciable quantities of carbohydrate and protein. To obtain further information about the chemical nature of this hemagglutinin, the substance obtained by acetone treatment of \textit{H. aegyptius} cells was most thoroughly examined because of apparent purity, serological reactivity, and absence of nitrogenous residues.

Descending paper chromatography was used to identify the component monosaccharide units of the extracted hemagglutinin. Serologically active extracts, by both the diethylene glycol and acetone methods, were hydrolyzed in HCl and spotted with glucose, mannose, galactose, and N-acetylgalcosamine standards on Whatman no. 1 paper. Butanol-ethyl alcohol-water (4:1:5) served as a solvent system. After approximately 24 hr, or until the solvent front had traveled the entire length of the paper, the spots were revealed with silver nitrate solution and identified by their \textit{Rf} values or by comparison with the migration of the standards.

In an attempt to determine the presence and nature of any predominant amino acids, corresponding chromatograms were prepared and developed with ninhydrin reagent. A quantitative examination of this extract was made for the following: uronic acid, Dische (6); total carbohydrate, Devor (2); total nitrogen, Koch-McMeekin (4); hexosamine, Elson-Morgan (6); sialic acid, Svennerholm (6); phosphorus, Fiske-Subbarow (6).

The total percentage of lipid of the acetone-extracted hemagglutinin was also determined by extract-
ing quantities of dried material with chloroform-methanol (2:1).

RESULTS

While attempting to modify the extraction procedure of Morgan (9) by using diethylene glycol, it was noted that acetone, used originally as a drying agent, could be applied to extract hemagglutinin from *H. aegyptius*. This serologically active material was soluble in both acetone and diethylene glycol. After overnight treatment of cell masses with diethylene glycol, the extract filtrate was clear. Dialysis of this against distilled water resulted in the precipitation of a water-insoluble, serologically active fraction that, upon suspension, directly hemagglutinated human red blood cells. Similarly, overnight treatment of cells with acetone removed even larger quantities of cell-bound hemagglutinin. The acetone solution was completely clear and exhibited no evidence of colloidal material after filtration. However, dialysis of this solvent against distilled water again resulted in the precipitation of serologically active hemagglutinin.

Since Davis, Pittman, and Griffiths (1) suggested that a significant distinction between *H. aegyptius* and *H. influenzae* was the capacity of the former to directly hemagglutinate human red cells for comparative purposes, the latter was also extracted with acetone. Extracts of this organism failed to exhibit any evidence of hemagglutination by established hemagglutination tests. Table 1 illustrates the typical hemagglutination titers obtained by extraction procedures employed in this study in comparison to values obtained by using unextracted *H. aegyptius* cells.

Antisera prepared to red cells modified with

<table>
<thead>
<tr>
<th>Method of extraction</th>
<th>Hemagglutination titers*</th>
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<tr>
<td></td>
<td>2</td>
</tr>
<tr>
<td>Diethylene glycol</td>
<td></td>
</tr>
<tr>
<td>Acetone</td>
<td></td>
</tr>
<tr>
<td>Alkaline hydroxide</td>
<td></td>
</tr>
<tr>
<td>Saline**</td>
<td></td>
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<tr>
<td>Unextracted bacterial</td>
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* Titers expressed as reciprocals of extract dilutions.

** See reference 4.

isotonic acetone-extracted hemagglutinin, red cells treated with alkaline-extracted hemagglutinin obtained in earlier studies, intact bacterial cells, and isotonic acetone extract were tested for their capacity to inhibit hemagglutination by each of the original antigenic preparations. Typically, any of the above antisera, with the exception of that prepared by immunization with isotonic acetone extract, would inhibit hemagglutination. The active sera consistently inhibited in titers ranging from 1:640 to 1:5,120. The antisera to red cells modified with isotonic alkaline extract were least effective in this capacity. It is significant that each antisera also inhibited hemagglutination by hemagglutinin extracted by each method. The results of this inhibition study are presented in Table 2.

The results of cross-desorption with the various antigenic preparations indicated that no differences existed in the antibody content of the sera, inasmuch as either homologous or heterologous adsorption removed all pertinent antibody.

The antigenic similarity of hemagglutinin extracted by the various methods was clearly demonstrated by diffusing hemagglutinin extracted by alkaline saline against antisera to red cells modified with acetone extract, intact bacterial cells, and red cells modified with alkaline extract. Antiserum from rabbits immunized with intact cells of *H. influenzae* and diffused against the soluble alkaline extract of *H. aegyptius* revealed no line of identity.

The comparison of hydrolyzed acetone and diethylene glycol-extracted hemagglutinin with reference sugars on descending paper chromatograms indicated the occurrence of glucose, mannosamine, and N-acetimylosamine in each serologically active extract. Corresponding chromatograms which were processed with ninhydrin reagent failed to indicate the presence of amino acids in any detectable quantity.

Of the positive quantitative chemical determinations, acetone extracts were shown to contain hexamiosamine but no uronic acid. Determination of the percentage of lipid in this extract was calculated by using the difference between the extracted weight and the original sample weight. Table 3 shows the per cent composition of the extracted hemagglutinin with respect to the various analytical tests.

DISCUSSION

A modification of the method described by Morgan (9) in which diethylene glycol was used to obtain specific antigenic substances from *Shigella* was applied in this study. This procedure revealed that the hemagglutinin of *H. aegyptius*...
could be extracted by either diethylene glycol or by acetone. The water-insoluble extracts recovered by either method were shown to be essentially nitrogen-free in contrast to that obtained previously by alkaline extraction (13). These extracts were also unaffected by changes in pH and were not soluble at high pH as were the alkaline extracts.

Antisera against intact bacterial cells, or red cells modified with either alkaline or acetone extract, inhibited hemagglutination by homologous and heterologous preparations. These results indicate the presence of essentially identical hemagglutinin obtained by each extraction method. The lines of identity expressed between antisera to acetone-extract-modified and alkaline extract-modified red cells and to antiserum to unextracted *H. aegyptius* cells support the similarity of the basic content of each extract fraction.

The fact that amino acids were not detected in the material extracted by acetone indicated that protein, at least at a readily detectable level, was not involved in the hemagglutination phenomenon exhibited by this organism.

In view of the findings by Ivler (5), who maintained that the hemagglutination test is not adequate in separating *H. aegyptius* from *H. influenzae*, it should be emphasized that alkaline extracted saline extracts of nontype-specific *H. influenzae* revealed no precipitin line when diffused against antisera to intact *H. aegyptius* cells or antiserum to red cells modified with extracts thereof. The former workers, however, employed type-specific *H. influenzae* strains collected from cases of purulent meningitis and grew their cultures in a more elaborate medium. During our investigation, the capacity of *H. aegyptius* to directly hemagglutinate human erythrocytes proved entirely adequate as a method for distinguishing it from the closely related nontype-specific *H. influenzae*. Orfila and Courden (11) also demonstrated a satisfactory correlation between the hemagglutination phenomenon of *H. aegyptius* and allied tests which distinguish it from *H. influenzae*.

Still no obvious answer presents itself to explain just why this capacity occurs. Certainly no unique compounds could be detected. Perhaps the answer as to why this capacity exists lies in structure or spatial relation of the intact polysaccharide molecule. Little is known about the role of lipid in somatic networks. Evidence as to the role of lipid in certain serological reactions has been slow in accumulation but has been implied by many workers (8, 10). The recent investigation by Tsumita and Ohashi (14) clearly indicates the importance of lipid. These workers, while studying a serologically specific polysaccharide containing, mannose, arabinose, and glucose from *M. tuberculosis*, were aware that this compound did not sensitize red cells as detected by passive

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**Table 2. Inhibition of hemagglutination by homologous and heterologous antisera**

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Antiserum against</th>
<th>Titer of hemagglutination inhibition</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>160&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Intact bacterial cell</td>
<td>Acetone-RBC&lt;sup&gt;b&lt;/sup&gt; NaOH-RBC <em>H. aegyptius</em></td>
<td>-</td>
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<tr>
<td></td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>Acetone extract</td>
<td>Acetone-RBC NaOH-RBC <em>H. aegyptius</em></td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>Diethylene glycol extract</td>
<td>Acetone-RBC NaOH-RBC <em>H. aegyptius</em></td>
<td>-</td>
</tr>
</tbody>
</table>

<sup>a</sup> Titer expressed as reciprocals of serum dilutions.

<sup>b</sup> Red blood cells.

<sup>c</sup> Reactivity of antigens in saline.

**Table 3. Chemical analysis of hemagglutinin**

<table>
<thead>
<tr>
<th>Test</th>
<th>%</th>
<th>Indication</th>
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<tbody>
<tr>
<td>Micro-Kjeldahl</td>
<td>1.0</td>
<td>Total nitrogen</td>
</tr>
<tr>
<td>Elson-Morgan</td>
<td>1.0</td>
<td>Hexosamine</td>
</tr>
<tr>
<td>Devor</td>
<td>57.0</td>
<td>Total carbohydrate</td>
</tr>
<tr>
<td>Chloroform-methanol</td>
<td>42.0</td>
<td>Lipid</td>
</tr>
<tr>
<td>Dische</td>
<td>Negative</td>
<td>Uronic acid</td>
</tr>
<tr>
<td>Svennerholm</td>
<td>Negative</td>
<td>Sialic acid</td>
</tr>
<tr>
<td>Fiske-Subbarow&lt;sup&gt;b&lt;/sup&gt;</td>
<td>6.0</td>
<td>Phosphorus</td>
</tr>
</tbody>
</table>

<sup>a</sup> Prepared by acetone treatment.

<sup>b</sup> Per cent phosphorus of lipid fraction.
hemagglutination techniques. After partial acylation with palmitoyl chloride, the resultant lipopolysaccharide sensitized erythrocytes and exhibited antigenic specificity.

In view of the high lipid content found in the acetone-extracted hemagglutinin of *H. aegyptius*, further investigations similar to that just described may reveal the mechanism involved in direct microbial hemagglutination.

**LITERATURE CITED**