STUDIES ON THE GONOCOCCUS

II. PROPERTIES OF AN ANTIGENIC FRACTION ISOLATED FROM CELL-FREE GONOCOCCAL BROTH SUPERNATANTS

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Serologically active substances have been isolated from the gonococcus as well as from the broth in which the organism has been grown, but as yet not all of them have been chemically defined. Several investigators (DeChristmas, 1897; Herrold, 1927; Clark, Ferry and Steele, 1931; and Wolfenstein and Pieper, 1931) have reported that broth in which the gonococcus has been grown elicits a typical dermal reaction in patients with gonococcal infection but not in persons without evidence of the disease. These findings have been interpreted as evidence that the gonococcus elaborates an immunologically active substance during growth in a broth medium. Recently, Rossett (1939), working with gonococcal bouillon filtrates, separated a fraction which possessed antigenic properties similar to those noted by previous workers. He considered this material to be a carbohydrate, a conclusion which was not supported by quantitative chemical proof.

By fractionation procedures similar in a number of respects to those employed by Rossett, we have isolated a fraction from gonococcal broth supernatants which was antigenic and toxic for laboratory animals. The present report describes the isolation and properties of this fraction, which is proteic in nature. No evidence was found to indicate that we were dealing primarily with a carbohydrate.

MATERIALS AND METHODS

Preparation of fraction

Seven strains of Neisseria gonorrhoeae, which had been under artificial cultivation for from 1 month to 34 years, were selected for the study. The organisms were grown in 300-ml. flasks containing 100 ml. of Douglas's broth to which 0.5 per cent each of sodium phosphate and sodium nitrate had been added. After 3 days of incubation at 37°C, the cells were removed by centrifugation. The broth supernatants were concentrated ten-fold in vacuo at temperatures not exceeding 20°C., and the broth constituents of low molecular weight were then separated by dialysis in the cold. Small amounts of nucleoprotein were removed from the broth concentrate by precipitation at pH 4.5. The resulting supernatant, when brought to neutrality and dialyzed against 25-per cent alcohol
containing 0.85-per cent sodium chloride ("saline") at 4°C, yielded a fraction possessing the specific antigenic properties of the unconcentrated cell-free broth. The method of cold alcohol precipitation was similar to that employed by Cohn (1941) for the fractionation of plasma proteins. The precipitate so obtained was washed once with the alcohol sodium-chloride medium and the alcohol removed either in vacuo or by dialysis. The antigenic activity of the fraction was tested by its capacity to bind complement in the presence of specific antigonococcal lapine and human sera. The alcohol-precipitated fraction was further purified by means of ammonium sulfate fractionation. The specificity of the fraction so obtained as well as the unpurified fractions were determined by means of the complement-fixation test according to the technique described by Price (1933).

**Determination of toxicity of purified fraction**

The toxicity of the broth fraction (MLD_{100}) was determined by the intra-abdominal injection of white mice weighing 20–22 gm. and by the intravenous injection of albino rabbits weighing 2.5–3 kg.

**Preparation of antisera**

Antisera for testing the specificity of the purified fraction were prepared from strains of *Neisseria gonorrhoeae*, *Neisseria intracellularis*, *Neisseria catarrhalis*, and *Neisseria sicca*. Thrice-washed organisms harvested from the cultures used for the preparation of the fraction were killed with 0.5-per cent formaldehyde and washed three times with "saline." Albino rabbits weighing 2.5–3.5 kg. were injected intravenously with gradually increased quantities of cells (0.07–0.7 mg. bacterial nitrogen). Injections, totaling 16, were made 4 times a week for 4 weeks. The rabbits were bled 5 days after the last injection. Antisera were also prepared against the purified fraction in the form of an alum-precipitated formalized suspension. Not more than 0.05–0.5 mg. of protein nitrogen could be injected because of the toxicity of the antigen. The course of the injections was similar to that described above.

**Electrophoretic analysis**

Fractions purified by precipitation with 0.4 saturation ammonium sulfate were subjected to electrophoretic examination in a single section cell Tiselius apparatus employing the Longsworth scanning method to record the electrophoretic pattern. Observations were made on two fractions freshly prepared, and on another that had been stored at 4°C. for 1.5 years. Solutions, containing from 0.67 to 1.00 mg. of protein nitrogen per ml. in veronal buffer, pH 8.46, ionic strength, μ, 0.1, were employed for the tests.

**RESULTS**

A precipitate, light brown in color, was recovered from gonococcal broth supernatants by alcohol precipitation in the cold, a method which has been

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1 The electrophoretic examination was carried out by Dr. E. L. Alling of the Department of Radiology, University of Rochester School of Medicine and Dentistry.
shown to produce little change in the nature of proteic material (Cohn, 1941). The yield of different preparations of the alcohol precipitate ranged from 95 to 135 mg. per liter of unconcentrated broth supernatant. The yield of precipitate from similarly treated uninoculated Douglas's broth was 15 mg. per liter. A significant difference was observed in the optical rotation of the fraction recovered from the gonococcal broth supernatants and from the broth control. The values for optical rotation, calculated from the formula, $\left[\alpha\right]_D^{23} \times 100/mg. N$, were from $-38^\circ$ to $-41^\circ$ for the supernatant and $-66^\circ$ for the broth control.

Ammonium sulfate fractionation of the alcohol precipitate effected the removal of substantial amounts of inactive material. The fraction possessing maximal complement-fixing activity per mg. of nitrogen was obtained in greatest yield at 0.4 saturation ammonium sulfate (see table 1). The yield of the purified fraction amounted to 55 per cent of the alcohol precipitate, and each preparation showed uniformity in this respect. Attempts to precipitate this fraction with ammonium sulfate from either dilute or concentrated broth supernatants yielded a highly pigmented, gummy product unsuitable for serologic tests.

### TABLE 1

**Complement-fixing activity of gonococcal broth antigen fractionated by precipitation with (NH$_4$)$_2$SO$_4$**

<table>
<thead>
<tr>
<th>SULFATE FRACTION, SATURATION (NH$_4$)$_2$SO$_4$</th>
<th>PRECIPITATE RECOVERED IN SULFATE FRACTION</th>
<th>DEGREE OF COMPLEMENT-FIXING ACTIVITY$^{*}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>per cent</td>
<td>per cent</td>
<td></td>
</tr>
<tr>
<td>33</td>
<td>48</td>
<td>++++</td>
</tr>
<tr>
<td>40</td>
<td>55</td>
<td>++++</td>
</tr>
<tr>
<td>33-50</td>
<td>23</td>
<td>+++</td>
</tr>
<tr>
<td>40-100</td>
<td>40</td>
<td>-</td>
</tr>
</tbody>
</table>

$^{*}$ ++++ indicates that 0.1-0.2 mg. of the fraction bound 3-5 MHD of guinea pig complement in the presence of specific antiserum.

**Chemical analysis**

Analytical data on representative preparations of the fraction purified by 0.4 saturation ammonium sulfate are recorded in table 2. The results are typical of those given by a proteic or protein-like material containing variable amounts of carbohydrate.

**Stability**

Physiologic "saline" solutions of the fraction preserved with a 1:10,000 dilution of merthiolate at 4°C. were stable for at least one year. The fraction obtained in the form of a white amorphous powder by precipitation from solution with acetone in the cold showed little loss in complement-fixing activity upon re-solution in "saline." Precipitation of the fraction from solution with 1.5 volumes of alcohol at room temperature resulted in diminished solubility, toxicity, and complement-fixing activity. Renaturation, with restoration of these properties, could be effected in part by resuspension in "saline." Protamine sulfate at pH 5.4 removed the purified fraction from solution.
Immunologic activity

Immunization of rabbits with the alum-precipitated formalinized fractions yielded antisera of low antibody content (0.2–0.3 mg. antibody nitrogen per ml.). The sera failed to precipitate their homologous fractions but agglutinated suspensions of gonococci as well as precipitates of gonococcal nucleoprotein. Furthermore, the lethal effects of the fraction were not neutralized with its specific antiserum when tested in mice.

The fraction was found to be a suitable antigen in the complement-fixation test for gonococcal infection. An amount of 0.1 mg. bound from 3 to 5 minimal hemolytic doses (MHD) of guinea pig complement in the presence of serum from patients with gonococcal infection, as well as in the presence of antigonococcus lapine serum. All preparations of the fraction, irrespective of the strain of gonococcus from which they were obtained, exhibited cross fixation to varying degrees in antisera prepared against Neisseria intracellularis, Neisseria catarrhalis, and Neisseria sicca.

Action of enzymes

The proteic nature of the fraction was further established by treatment with proteolytic enzymes. The complement-fixing property of the purified fraction was gradually destroyed by the action of either pepsin at pH 4.5 or pancreatin at pH 7.5 at a temperature of 37°C. Development of anticomplementary properties was noted in the case of the pepsin-treated antigen. The fraction was then subjected to incomplete proteolytic enzymic action in an endeavor to eliminate group-specific reactions with other members of the genus "Neisseria. It was observed, however, that the partial enzymic action failed to eliminate uniformly the group specific reactions of the gonococcal antigen with antisera prepared from Neisseria intracellularis, Neisseria catarrhalis, and Neisseria sicca, as measured by complement-fixation methods.

* The merthiolate was supplied by Eli Lilly and Company, Indianapolis, Indiana.
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Toxicity for experimental animals

The fraction was markedly toxic for mice and rabbits. The MLD100 for 22-gm. white mice was 0.016-0.10 mg. protein nitrogen; for 2.5-3.5-kg. albino rabbits, 0.5-0.6 mg. protein nitrogen.

Electrophoretic behavior

Photographs taken 3 hours and 10 minutes after electrophoresis of three lots of the ammonium sulfate fractions produced a pattern which consisted of a main peak (electrophoretic mobility, $\mu$, $4.35 \times 10^{-5}$ at pH 8.46) gradually sloping into a lower, less well-defined peak ($\mu$, $2.12 \times 10^{-5}$). The pattern was interpreted as indicating non-homogeneity although the protein-like molecules were similar in electrophoretic behavior (fig. 1).

A second observation was made on one sample and separation of the fast from the slow component was accomplished. Complement-fixation tests employing the two components showed that the major, fast component possessed antigenic activity but that the minor, slow component was devoid of it. Chemical analyses of the slow component demonstrated that from a 4- to 5-fold increase in carbohydrate had occurred.

DISCUSSION

Most of the biochemical fractions of the gonococcus that have been described (Warden, 1915; Boor and Miller, 1934; Miller and Boor, 1934; Casper, 1937) have been tested immunologically, but have received scant chemical definition. In the present instance an attempt has been made to characterize the fraction isolated from gonococcal broth supernatants chemically as well as immunologically.

Studies on the immunologic and toxic reactions of the purified fraction indicate that it is a degraded protein derived from the nucleoprotein of the gonococcal cell. The immunologic similarity of the two proteins is shown by the precipitation of gonococcal nucleoprotein by antisera prepared against the broth fraction. In experimental animals, furthermore, the fraction produces "toxic" effects characteristic of gonococcal nucleoprotein (Boor and Miller, 1934). Our ob-
servations indicate that the purified fraction is from 3 to 4 times as toxic as cellular nucleoprotein when compared on a nitrogen basis. That the fraction is not a complete nucleoprotein, however, is proved by its low analytical values for phosphorus (table 2), which indicate the presence of little or no nucleic acid in the molecule. Moreover, this fraction served as a poor antigen in rabbits, giving rise to antisera of low antibody content (0.2 mg. antibody N/ml.). In addition, when such sera were mixed with the fraction, no precipitate was observed. The latter two facts suggest that degradative changes other than loss of nucleic acid have occurred in the formation of the broth fraction from nucleoprotein of the cell.

The comparatively large amount of the antigen in the supernatant from 3-day cultures was striking. Its production on a nitrogen basis was, in general, equal to that of the cells harvested from the broth. Of special interest is the fact that this soluble fraction could be recovered only from the gonococcal broth supernatant. Every attempt to isolate the material from the cells was unsuccessful. A similar fraction has been obtained from cell-free broth supernatants of cultures of Neisseria intracellularis, Neisseria catarrhalis, and Neisseria sicca, a result which might be anticipated owing to the close serologic relationship of these species. The electrophoretic separation of the purified broth fraction into a main, fast-moving component consisting of antigenically active protein, and into a secondary slowly-moving component, rich in carbohydrate but antigenically inert, showed that further purification of the fraction is possible by electrophoresis.

SUMMARY

1. A protein-like fraction, possessing "toxic" and antigenic properties, was separated from broth cultures of Neisseria gonorrhoeae after removal of the cells by centrifugation.

2. The fraction appeared to be a protein degradation product derived from the nucleoprotein of the cells. This conclusion is based upon its destruction by proteolytic enzymes, by reversible loss of complement-fixing activity after treatment with protein denaturants, by its peculiar immunologic behavior in specific antisera, and finally, by chemical analyses.

3. The purified fraction fixed complement in the presence of sera from rabbits immunized with Neisseria gonorrhoeae and of sera from patients with gonococcal infection.

4. The toxicity of the fraction for laboratory animals was greater than that of nucleoprotein of the cells. The MLD100 of several preparations varied from 0.1 to 0.7 mg. for white mice weighing from 20 to 22 gm.

5. The major component of the purified fraction consisted of molecules with similar electrophoretic behavior and antigenic activity. The minor component was rich in carbohydrate and non-antigenic.

REFERENCES

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Cohn, E. J. 1941 The properties and functions of the plasma proteins with a consideration of the methods for their separation and purification. Chem. Revs., 28, 395-417.


