

THE SEROLOGICAL CLASSIFICATION OF
NEISSERIA GONORRHOEAE

I. Isolation of the Outer Membrane Complex Responsible for Serotypic
Specificity*

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Gonorrhoea has become one of the most prevalent bacterial infectious diseases reported by the public health services of many countries. The factors contributing to the pandemic status of this disease are numerous. The most notable of these are, firstly, the gonococcus has a short incubation period with a concomitant high degree of transmissibility and, secondly, there appears to be no lasting immunity to the gonococcus. The latter may reflect the existence of distinct antigenic populations of the organism. Consequently, an important key to the stemming of the spread of this ubiquitous pathogen would be the knowledge of the existence of distinct serotypes, enabling the epidemiologist to discern patterns of gonococcal transmission.

Although *Neisseria gonorrhoeae*, the etiological agent of gonorrhoea, has been known since its identification by Neisser in 1879, no standard serological classification of the gonococcus into types or groups analogous to those described for the streptococcus by Lancefield (1) or for the Salmonella (2) has been developed. Early investigators, using complement fixation (3, 4), demonstrated the presence of a thermostable common gonococcal protein antigen in addition to several "type-specific" and "strain-specific" partial antigens. However, Casper (5) and Uroma (6) considered a carbohydrate moiety as the type-specific fraction, but the investigations of Stokinger (7) failed to confirm these observations. Lipopolysaccharide has also been implicated as a potential serological typing agent (8, 9); Maeland and Kristoffersen (10) define a series of carbohydrate determinants, alpha-antigens, from the gonococcal cell wall. They suggested that these determinants may be useful for subgrouping the gonococci in a manner analogous to the O antigens of the Enterobacteriaceae. Apicella and Allen (11, 12) extended these observations to differentiate the alpha-antigens described by Maeland into two acidic polysaccharides which differentiated two antigenically distinct populations of *N. gonorrhoeae* from 10 randomly selected gonococcal strains. Hutchison (13), using the Lancefield hydrochlo-

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ric acid extraction procedure (1), examined 181 strains of *N. gonorrhoeae* and was able to classify 143 strains into five distinct serological groups. She found that repeated subculture caused the gonococcus rapidly to lose its serospecificity. Similarly, Quinn and Lowry (14) categorized 208 gonococcal isolates into nine serologically distinct groups. Tramont et al. (15) demonstrated antigenic differences and similarities among strains of gonococci using a bacteriocidal assay involving rabbit antisera to surface protein antigens and lipopolysaccharides. These investigators' results were similar to those of Glynn and Ward (16) who also used a bacteriocidal assay to divide 60 gonococcal strains in four broad overlapping groups. An antigenic relationship between *Neisseria meningitidis*, *Neisseria catarrhalis*, and *N. gonorrhoeae* has long been known and has been demonstrated by agglutination (8), precipitation (17), and complement fixation tests (18). A serological relationship between *N. gonorrhoeae* and *Neisseria sicca* was demonstrated using quantitative agglutination techniques (19). Using fluorescein-conjugated antigenococcus globulins, Deacon et al (20) demonstrated cross-reactions between *N. meningitidis* and *N. gonorrhoeae*. With this method, weak reactions were also noted with *N. catarrhalis*. These observations were confirmed by Danielsson (21) who also noted that *N. sicca*, *Neisseria flava*, and *Neisseria flavescens* reacted with antigenococcal globulin. Recent investigations, most notably those of Carifo and Catlin (22) and LaScolea and Young (23), have dwelt upon the feasibility of auxotyping, while others (24, 25), though unsuccessful, have probed the possibility of bacteriocins as potential candidates for gonococcal serotyping.

The isolation of pili as a discrete protein antigen of the virulent colonial types, T₁ and T₂, by Buchanan et al. (26) prompted the analysis of pili as having serotypic specificity. Very recently, it has been demonstrated there is antigenic heterogeneity among gonococcal pili (27, 28). However, all of the above approaches reinforced the concept that the gonococcus was polyantigenic and thus evaded classification into distinct serological types.

It is a well recognized fact that the bacterial cell wall and its surface constituents are important in determining the pathobiological characteristics of many eubacteria. The roles played by surface components extend from the determination of interactions with host phagocyte cells to presentation of antigenic substances against which protective antibodies can be formed. Although our understanding of the pathobiology of the gonococcus is far from complete, evidence suggesting the importance of the gonococcal surface is emerging. The outer membrane of gram-negative bacteria contains protein and lipopolysaccharide in addition to lipids. The lipopolysaccharides have been the basis of a serotyping system for *Escherichia coli* (29) and *Salmonella* (2). However, outer membrane proteins have not been fully exploited as potential serological agents. Other than Pili, no one to date has isolated a specific antigen of the gonococcal cell envelope which possesses unique characteristics permitting its use as a serologically discriminating agent. The work of Frasc and Gotschlich (30) on *N. meningitidis* demonstrated the presence of a protein type-specific antigen for group B meningococci. This protein resides in the outer membrane of the meningococcus and accounts for the bulk of the total protein of that membrane. Numerous investigators (31-33) have demonstrated that the outer membrane of gram-negative bacteria is unique in that it has a relatively simple protein spectrum with usually one or two protein species predominating; the cell envelope of the gonococcus is no exception.

In our initial studies (34, 35) on the membranes of the gonococcus, it was demonstrated that a polypeptide, the major protein, accounted for over 60% of the total protein of the outer membrane. We have extended our studies to demonstrate that this protein carries antigenic determinants permitting us to differentiate serotypes in a population of gonococcal isolates. The serotype

antigen proved to be an integral part of a high molecular weight lipoprotein-lipopolysaccharide complex representative of the outer membrane. This communication describes the procedures whereby we isolated the type-specific antigen and used it as the basis of a serotyping system for the gonococcus.

Materials and Methods

Bacteria and Media. *Neisseria gonorrhoeae* and other *Neisseria sp.* were obtained from several sources. Gonococcal strains 2686 and F62 were obtained from Dr. D. S. Kellogg, Jr., Center for Disease Control, Atlanta, Ga., as were samples of the nonpathogenic neisseria, *N. flava*, *N. sicca*, and *N. lactamica*. The number of strains isolated in the New York City area by ourselves was augmented by the generous contributions of Dr. Y. C. Faur, Public Health Laboratory Services, The City of New York, Department of Health, New York; Dr. Stephen Kraus of the Center for Disease Control, Atlanta, Ga.; and Dr. John Swanson, Department of Pathology, University of Utah College of Medicine, Salt Lake City, Utah. Strain M986 of *N. meningitidis*, group B, serotype 2, was kindly supplied by Dr. C. E. Frasch, Bureau of Biologics, Public Health Service, Food and Drug Administration, Bethesda, Md. Gonococcal isolates from Taiwan, Denmark, Ethiopia, Vietnam, the Philippines, and Seattle, as well as most strains isolated from known contact pairs and from patients having disseminated gonococcal disease, were from the collection of organisms maintained at the University of Washington, Seattle, Wash. *E. coli* was isolated by the author (KHJ) from a fecal sample.

Gonococcal strains obtained from patients after cultivation on Thayer-Martin selective gonococcal medium (36) were identified by gram stain, oxidase (37), and fermentation (38) reactions. Colonial types T₁, T₂, T₃, and T₄, as described by Kellogg et al. (39), were maintained by selective subculturing, preserved in Greaves freezing solution (40), and stored at -76°C or lyophilized after suspension in Greaves solution.

Bacteria were subcultured for 16 h in GC agar base supplemented with Isovitalex (Baltimore Biological Laboratories, Cockeysville, Md.) at 36°C in a candle extinction jar. For growth in liquid culture, approximately 2×10^8 bacteria were suspended in 200 ml trypticase soy broth, pH 7.3 (Baltimore Biological Laboratories) supplemented with 10 mM MgSO₄. After cells were grown at 35.5°C with constant agitation to late log phase, bacteria were harvested by centrifugation at 10,000 g for 10 min.

Extraction Procedure. Approximately 500 mg (wet weight) intact cells were suspended in 30 ml of 100 mM tris(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer, pH 8.0 with 200 mM sodium chloride and 0.002% (wt/vol) sodium azide added as preservative (TS buffer)¹. The suspension was incubated for 2 h at 45°C with vigorous agitation in the presence of glass beads, 6 mm in diameter. Cells were pelleted by centrifugation at 15,000 g for 20 min; the supernate was retained. Glass beads were washed with TS buffer to remove adsorbed material. This wash was centrifuged at 15,000 g for 20 min and the supernate retained. Supernates were combined and centrifuged for 2 h at 100,000 g; the resultant translucent pellet was resuspended in 500 μ l TS buffer. Resuspended pellets were applied to a 1.5 \times 30-cm Sepharose 6B column (exclusion limit, 4×10^6 daltons for globular proteins; Pharmacia Fine Chemicals, Piscataway, N. J.) equilibrated with TS buffer. Fractions eluted from the column were monitored for protein by absorbance at 280 nm or by the Folin reaction. Comparative sodium dodecyl sulfate (SDS)-polyacrylamide slab gel electrophoresis (PAGE) and immunological double diffusion in agar gels were used to monitor the separation of proteins. In addition, fractions eluted from a Sepharose 6B column were acidified by addition of 10% (vol/vol) acetic acid to pH 4.2. After standing for 1 h at 4°C, the acidified fractions were centrifuged at 30,000 g for 30 min, and the pellets were resuspended in 500 μ l 10 mM TS buffer, pH 8.5.

An alternate extraction procedure was employed using 200 mM lithium acetate buffer, pH 6.0 with 10 mM ethylene diamine-tetra acetic acid (LAE) as the extraction buffer. In addition, cells from the first extraction were reextracted in LAE buffer and processed as above.

¹ Abbreviations used in this paper: BSA, bovine serum albumin; KDO, 2-keto-3-deoxyoctonate; LAE, 200 mM lithium acetate buffer, pH 6.0 with 10 mM EDTA; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; TS, 100 mM Tris-HCl buffer, pH 8.0 with 200 mM NaCl.

Lipopolysaccharide Extraction. The procedure of Westphal and Jann (41) was used. Approximately 5 g wet weight of cells obtained from 3 liters of culture grown in trypticase soy broth supplemented with 0.25% (wt/vol) MgSO₄ were suspended in 100 ml distilled water followed by addition of 100 ml 90% liquid phenol (J. C. Baker Co., Phillipsburg, Pa.). The suspension was heated to 68°C for 20 min, cooled to 4°C, and centrifuged 5,000 g at 4°C for 15 min. The aqueous phase was aspirated and retained. To this was added 100 mg sodium acetate (J. C. Baker Co.) and 2 vol of cold (-20°C) acetone (J. C. Baker Co., reagent grade). The precipitate was harvested and washed twice with cold acetone. After the precipitate (lipopolysaccharide) was dissolved in distilled water it was dialyzed for 36 h against three changes of distilled water. The lipopolysaccharide was pelleted by centrifugation at 100,000 g for 2 h and then resuspended in distilled water and repelleted several times until the supernate gave an absorbance of < 0.05 at 260 nm. The lipopolysaccharide was resuspended in distilled water and lyophilized.

Radiolabeling of Proteins. When cultures were to be intrinsically labeled, approximately 5 μ Ci of each of the following isotopes were added to 100 ml of culture medium: uniformly labelled L-[¹⁴C]leucine (460 mCi/mmol) and L-[¹⁴C]tyrosine (304 mCi/mmol), or L-[4,5-³H]leucine (5 Ci/mmol) and L-[3,5-³H]tyrosine (48.2 Ci/mmol), all obtained from New England Nuclear, Boston, Mass. Radioactive fractions were added to 5.0 ml of Hydromix liquid scintillation fluid (Yorktown Research, New Hyde Park, N. Y.), and the radioactivity was determined in a refrigerated Packard Tri-Carb liquid scintillation counter. Counting efficiencies were 84% for ¹⁴C and 47% for ³H.

Polyacrylamide Electrophoresis. Proteins are analyzed electrophoretically by SDS-PAGE according to Weber and Osborn (42) and modified for comparative slab gel electrophoresis as described by Maizel (43). A 1% (wt/vol) SDS (Bio-Rad Laboratories, Richmond, Calif.), 10% (wt/vol) acrylamide (Eastman Kodak Co., Rochester, N. Y.), 0.025% (wt/vol) N,N'-methylenebisacrylamide (Eastman Kodak Co.) gel with a 1% (wt/vol) SDS, 5% (wt/vol) acrylamide, 0.0125% (wt/vol) N,N'-methylene bisacrylamide spacer gel was prepared in glass molds, 11.5 cm by 13.0 cm by 0.5 cm. Samples to be electrophoresed were boiled at 100°C for 1 min in a solution of 10 mM sodium phosphate, pH 7.0, 2% (wt/vol) 2-mercaptoethanol and 2% (wt/vol) SDS; after cooling, one-half volume of 0.05% (wt/vol) bromophenol blue in 80% (wt/vol) sucrose was added. 20 μ l of sample were usually applied to the gel slot. After electrophoresis at 80 mA for 5 h, the slab gel was immersed for at least 6 h in a fixing-staining solution of 0.25% (wt/vol) Coomassie Brilliant Blue R (Sigma Chemical Co., St. Louis, Mo.) dissolved in methanol-acetic acid-water (5:1:4). The gel was subsequently immersed in a destaining solution of methanol-acetic acid-water (1:1:8) to which had been added 20 g (dry wt) Bio-Rex RG 501-X8, reactor grade, mixed bed resin (Bio-Rad Laboratories) per 500 ml destaining solution. Gentle agitation at 60°C accelerated the destaining procedure. Molecular weights were estimated using bovine serum albumin (BSA), ovalbumin, pepsin, and cytochrome *c* as markers.

Chemical Procedures. Protein was determined by the method of Lowry et al. (44) using BSA (Metrix Armour Pharmaceutical Co., Chicago, Ill.) as standard. Lipopolysaccharide content was determined by measuring the amount of 2-keto-3-deoxyoctonate (KDO; Sigma Chemical Co.) released from samples by hydrolysis with 0.02 N H₂SO₄ at 100°C for 20 min. Portions were removed and analyzed for KDO by the thiobarbituric acid procedure of Osborn (45); the absorbance was read at 548 nm. Nucleic acid was determined spectrophotometrically at 260 nm.

Isolation of Outer Membrane. The fractionation of the cell envelope into inner and outer membrane was accomplished by isopycnic sucrose density centrifugation of osmotically lysed spheroplasts as described by Johnston and Gotschlich (34).

Antisera. Isolated outer membranes or isolated serotype antigens suspended in phosphate-buffered saline (PBS), pH 7.5 to a protein concentration of 10 mg/ml, were emulsified with complete Freund's adjuvant (Difco Laboratories, Detroit, Mich.) 1:1 vol/vol; 0.5 ml was injected subcutaneously between the scapulae into New Zealand red rabbits. On day 30, rabbits were reinjected intravenously with 500 μ g protein suspended in Hanks' PBS (Grand Island Biological Co., Grand Island, N. Y.). Whole organisms used for injection were harvested from a 16-h culture grown on GC agar base. Approximately 10⁸ cells were suspended in 5 ml Hanks' basal salts medium; 0.5 ml aliquots were frozen and subsequently used for intravenous injection every 3rd day for 3 wk. Animals were allowed to rest 10 days before test bleeding. If titers were not satisfactory, a second identical regime was initiated. Sera were heated at 56°C for 30 min, and 0.02% (wt/vol) sodium azide was added as a preservative.

Immunodiffusion. Standard microscope slides were mounted on plastic templates and covered with 1.0% Noble agar dissolved in 100 mM sodium phosphate buffer, pH 7.5 with 150 mM sodium chloride and 0.02% (wt/vol) sodium azide. After addition of antigen and antisera to appropriate wells, slides were incubated at 23°C for 24–48 h in a humidified chamber.

Precipitin lines were recorded after visual inspection or immunodiffusion plates were stained with Coomassie Brilliant Blue R and were photographed.

Results

Electrophoretic Profiles of Outer Membranes of Gonococcal Strains. When isolated outer membranes of several gonococcal strains were examined by SDS-PAGE, a single protein of apparent electrophoretic homogeneity predominated. These outer membranes had a relatively simple electrophoretic pattern as opposed to the multibanded pattern of the spheroplast membrane (Fig. 1). The outer membrane had nine protein bands, one of which was predominant. By comparison to proteins of known molecular weight, these polypeptides of Strain 2686 (N001), for example, had subunit mol wt of 100,000, 90,000, 86,000, 64,000, 56,000, 48,000, 34,500, 27,000, and 11,500 daltons. In all strains examined, the major polypeptide accounted for over 60% of the total outer membrane and was, hence, called the major outer membrane protein. When 14 strains of gonococci isolated in the New York City area were compared by comparative SDS-PAGE, they could be categorized into five classes according to the electrophoretic profiles of their isolated outer membranes (Fig. 1). These strains shared at least six proteins, but most noticeable was the variance in the subunit molecular weight values of the major outer membrane protein. The subunit molecular weight values of the major polypeptide of these five classes ranged between

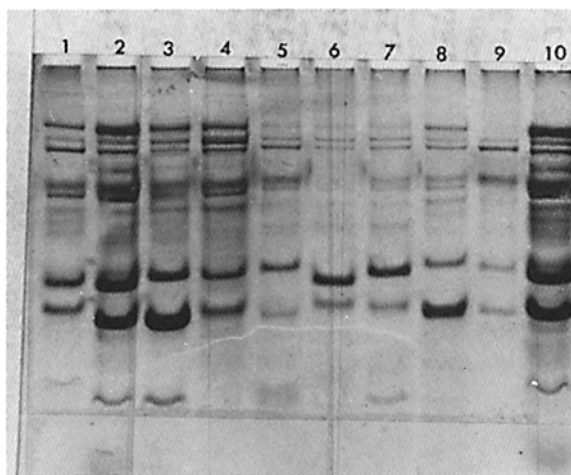


FIG. 1. Comparative SDS-PAGE of isolated spheroplast and outer membranes of *N. gonorrhoeae*. Wells 1 and 6 represent spheroplast and outer membrane, respectively, of strain N142; wells 2 and 7 represent spheroplast and outer membrane, respectively, of strain N100; wells 3 and 8 represent spheroplast and outer membrane, respectively, of strain N112; wells 4 and 7 represent spheroplast and outer membrane, respectively, of strain N137; wells 10 and 5 represent spheroplast and outer membrane, respectively, of strain N136. Samples were heated in 1% SDS at 100°C for 1 min and applied to gel. After electrophoresis, gel was stained with 0.025% Coomassie Brilliant Blue R.

38,000 and 32,000 daltons. On the basis of this molecular weight heterogeneity, 57 strains of gonococci isolated in the New York City area were grouped into seven electrophoretically distinct classes. Each class had a different subunit molecular weight value for the major outer protein. In addition, a second protein of lower subunit molecular weight than the major protein appeared to covary with the major protein. Table I summarizes the relationship between the major outer membrane protein and the associated minor protein for the seven electrophoretically distinct strains (A-G) isolated in the New York City area.

Isolation of Outer Membrane Complex Containing Major Outer Membrane Protein. To isolate this outer membrane protein for subsequent immunological analysis, several extraction procedures were investigated. Cell envelopes were extracted in anionic, cationic, and nonionic detergents; however, the difficulty of removing these agents after isolation discouraged further use.

When intrinsically labeled isolated outer membranes were incubated in 100 mM Tris-HCl buffer, pH 8.0 with 200 mM NaCl at 45°C for 2 h, approximately 38% of the total radioactivity was released into the supernate, 85% of which was eluted at the void volume of a Sepharose 6B column. SDS-PAGE demonstrated that this fraction contained the major outer membrane protein. However, the amount of total protein released was insufficient for subsequent repeated chemical and immunological analyses. This was not surprising, as it had been observed that during the spheroplasting procedure surface proteins are released into the spheroplasting medium. Thus, to increase yield of the complex containing the outer membrane protein, intact cells were extracted.

Gonococci harvested in late-log phase were extracted at pH 8.0 in 100 mM Tris-HCl buffer with 200 mM NaCl (TS buffer) for 2 h at 45°C. After cells were removed by centrifugation at 20,000 g, the supernate was concentrated by Diaflo

TABLE I
Subunit Molecular Weight of Proteins Present in STA Complex

Serotype	Major protein <i>daltons</i>	Minor protein <i>daltons</i>
A	34,500	22,000
B	32,000	27,000
C	38,000	26,000
D	36,500	27,500
E	38,000	25,000
F	37,000	22,000
G	33,000	27,500
H	32,000	24,000
N	34,000	29,500
R	32,500	22,000
S	34,000	23,000
T	37,500	27,500
U	34,000	28,000
V	33,500	24,000
W	39,000	31,000
X	37,000	26,000

STA, serotype antigen complex.

PM-10 membrane filtration. The concentrate was resolved by gel filtration on Sepharose 6B into three distinct fractions (Fig. 2). In order of elution, they were Fractions I, II, and III. SDS-PAGE of these fractions demonstrated that extracted membrane fragments were differentially resolved by gel filtration on Sepharose 6B (Fig. 3). For strain N001, the void volume (Fraction I) contained predominately the outer membrane complex containing the 34,500 daltons poly-peptide. Fraction II contained various proteins that had higher subunit molecular weights, ranging from 48,000 to 100,000 daltons. Fraction III was primarily nucleic acid. The extracts of several strains gave similar chromatographic profiles. However, this procedure using TS buffer favored the release of polypeptides that were common to all strains, that is, those proteins having subunit mol wt of 48,000-100,000 daltons. The membrane complex enriched in the major outer membrane protein was but a small fraction of the total protein released (Fig. 2).

A second extraction procedure was examined. Intact organisms harvested at late-log phase were extracted in LAE buffer at 45°C for 2 h with constant agitation. This mild extraction procedure caused minimal lysis of the cells. Periodic examination by electron microscopy during the 2-h incubation period indicated that the gonococci remained largely intact as the outer membrane gradually peeled away in the form of small vesicles. The integrity of the cell envelope was retained after a second extraction of 2 h at 45°C. However, after this period, the cells began to lyse and by 6 h 90% of the organisms had lysed. After cells had been pelleted by centrifugation and the supernate concentrated by centrifugation at 100,000 *g*, the pellet was resuspended in LAE buffer and chromatographed on Sepharose 6B equilibrated in the same buffer. Fig. 4 illustrates the SDS-PAGE of four gonococcal strains extracted as above having different outer membrane profiles. When the first extract was chromato-

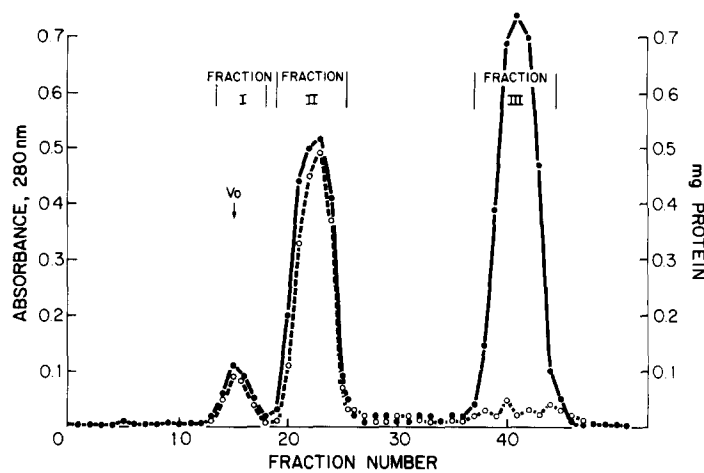


FIG. 2. Elution profile of 100 mM Tris buffer, pH 8.0 with 200 mM NaCl (TS) extract of *N. gonorrhoeae*, serotype A. 100,000 *g* pellet was applied to a Sepharose 6B column and eluted with TS buffer. Absorbance at 280 nm [●—●]; mg protein [○—○] determined by Folin reagent and read at 780 nm. V_0 ↓ represents the void volume of the column as measured with Blue Dextran.

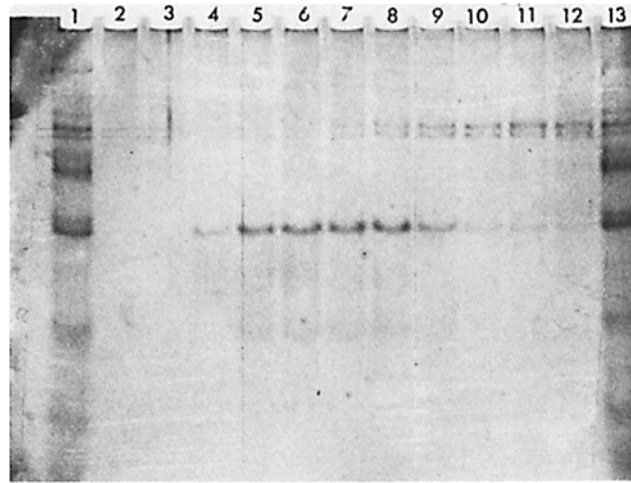


FIG. 3. SDS-PAGE of fractions eluted from Sepharose 6B column. 100 mM Tris buffer pH 8.0 with 200 mM NaCl extract of *N. gonorrhoeae*, serotype A. Wells 1, 13 = unfractionated 100,000 g pellet; well 2 = fractions 11 + 12; well 3 = fraction 13; well 4 = fraction 14; well 5 = fraction 15; well 6 = fraction 16; well 7 = fraction 17; well 8 = fractions 18 + 19; well 9 = fractions 20 + 21; well 10 = fractions 22 + 23; well 11 = fractions 24 + 25; well 12 = fractions 26 + 27. Elution profile depicted in Fig. 2. The samples were solubilized in 1% SDS at 100°C for 1 min and applied to the gel. After electrophoresis, the gel was stained with 0.025% Coomassie Brilliant Blue R.

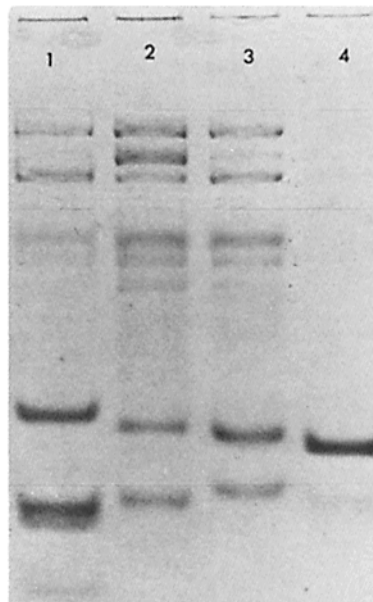


FIG. 4. Comparative SDS-PAGE of four strains of *N. gonorrhoeae* extracted in 200 mM lithium acetate buffer, pH 8.0 and 10 mM EDTA (LAE) for 2 h at 45°C. Extracts were pelleted at 100,000 g. Well 1 = serotype F; well 2 = serotype V; well 3 = serotype G; well 4 = serotype H. The different preparations were solubilized in 1% SDS at 100°C for 1 min and applied to gel. The gel was stained with 0.025% Coomassie Brilliant Blue R after electrophoresis.

graphed, two fractions were observed (Fig. 5). The major outer membrane protein was eluted at the void volume of this column. As indicated by its exclusion from a Sepharose 6B column, this protein existed as part of a large membrane complex greater than 4×10^6 daltons. It was a representative portion of the outer membrane; not only was it chemically similar to isolated outer membrane, in that it had 1.17 mg lipopolysaccharide to each mg of protein, but it banded in sucrose at the same buoyant density, $\rho^{\circ} = 1.22 \text{ g/cm}^3$. Other membrane proteins were eluted at a V_e/V_o ratio greater than one; these polypeptides were eluted at the same V_e/V_o value and had identical mobilities on SDS-PAGE as the proteins eluted as Fraction II of the TS-buffer extraction. These proteins had larger subunit molecular weights, but, as indicated by their inclusion in the Sepharose 6B column, were of smaller aggregate or vesicular size than the major outer membrane protein. When the product of second extraction was chromatographed, only one peak was observed at the void volume (Fraction I). Quantitative studies using cells labeled with radioactive leucine and tyrosine indicated that extraction with LAE buffer yielded at least five times more major outer membrane protein than extraction with TS buffer (Table II).

Addition of 10% (vol/vol) acetic acid to the fraction eluted at the void volume (Fraction I) such as to lower the pH to 4.2, precipitated the complex. This precipitate had a higher buoyant density in sucrose than the native isolated outer membrane; a value of $\rho^{\circ} = 1.25 \text{ g/cm}^3$ was obtained. Analysis demonstrated that there was a decreased amount of lipopolysaccharide. There were 0.72 mg lipopolysaccharide per mg protein. Thus, extraction of intact cells by incubation in 200 mM lithium acetate buffer, pH 6.0 with 10 mM EDTA, followed by chromatographic separation on Sepharose 6B and isoelectric precipitation of the membrane complex eluted at the void volume afforded relatively pure serotype

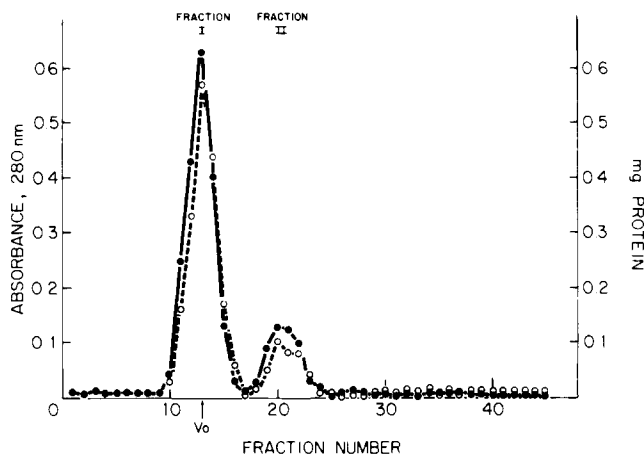


FIG. 5. Elution profile of 200 mM lithium acetate buffer, pH 6.0 with 10 mM EDTA (LAE) extract of *N. gonorrhoeae*, serotype A. 100,000 g pellet was resuspended in LAE buffer, applied to a Sepharose 6B column, and eluted with LAE buffer. Absorbance at 280 nm [●-●]; protein concentration determined with Folin reagent [○-○] read at 750 nm; [V_o] represents void volume as measured with Blue Dextran (Pharmacia Fine Chemicals).

TABLE II
 Extraction of ^{14}C -labeled Serotype Antigen Complex from Whole
 Cells, Strain 2686, by either TS buffer or LAE buffer at
 45°C for 2 h

Extraction procedure	Percent total in- corporated*	Percent total as STA‡
Tris-HCl-NaCl, pH 8.0	7.2	2.4
LiAc-EDTA, pH 6.0	8.1	16.2

* Total amount of ^{14}C -labeled amino acids incorporated determined on sample after cell pellet was washed once with cold (4°C) PBS and resuspended in extraction buffer.

‡ Percent of total counts of precipitated serotype antigen complex (STA) by 10% (vol/vol) acetic acid at pH 4.2.

antigen in sufficient amounts to initiate immunological studies. Using this extraction procedure, 167 strains of gonococci isolated in the New York City area were analyzed and grouped into eight electrophoretically distinct classes (A-G, V). Each class had a different subunit molecular weight for the major outer membrane protein. Each representative major outer membrane polypeptide was associated with a distinct and characteristic secondary protein, as described above. Table I lists outer membrane protein mol wt for each class with respect to their major outer membrane protein and corresponding covariant or secondary protein. These classes were arbitrarily assigned an alphabetical code.

Antigenic Specificity of the Serotype Antigen Complex. Antisera were prepared in rabbits to whole cells and to isolated outer membrane fragments enriched in the major protein. The immunological reaction was assayed by double diffusion in agar-gels. When whole cell vaccines were used, there were several nonspecific cross-reactions. Unfractionated homologous extracts gave a complex precipitin pattern. Most prominent was a heavy precipitin line concave to the antigen well (Fig. 6). When fractions eluted from a Sepharose 6B column were analyzed sequentially against homologous antiserum, there was a distinct separation of immunologically reactive material. Fraction I, protein eluted at the void volume, gave one precipitin line concave to the antigen well, whereas Fraction II, containing proteins of higher molecular weight, but of smaller aggregate or vesicular size, developed precipitin lines midway between the antigen and antibody wells. Fraction I from a TS-buffer extract and a LAE-buffer extract were identical; similarly, Fraction II was immunologically identical whether the source was a TS-buffer extract or a LAE-buffer extract. When an extract of a heterologous strain, that is, a strain having an electrophoretically dissimilar outer membrane protein, was analyzed by double gel diffusion, the specificity of the reaction was remarkable. An extract of a heterologous strain formed no precipitin line adjacent to the antigen well, but demonstrated immunological identity with proteins eluted from a Sepharose 6B column as Fraction II. Extracts of the nonpathogenic *Neisseria sp.*, *N. lactamica*, *N. sicca*, and *N. flava* gave no immunological identity to any of the antisera prepared to the major outer membrane complex of nine serologically distinct gonococcal serotypes. There was some cross-reaction of Fraction II of *N. sicca* and *N. flava* with five antigenococcal antisera. tested. The precipitin lines were indicative of

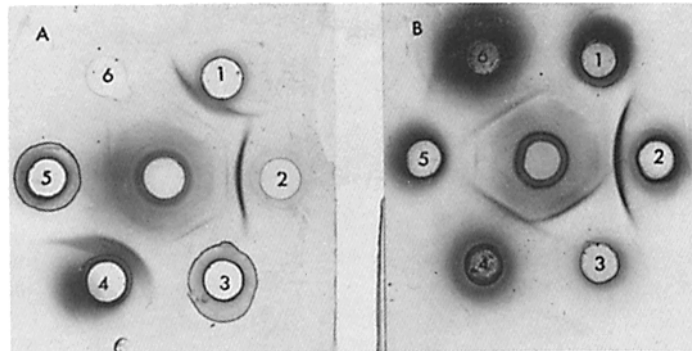


FIG. 6. (A) Immunodiffusion in agar gel of extracts of *N. gonorrhoeae* and *N. meningitidis*. Center well: antiserum prepared to 100,000 g pellet of *N. gonorrhoeae*, serotype A, extracted in LAE buffer. Antigen wells: (1) 100,000 g pellet, TS, extract, *N. gonorrhoeae*, serotype A; (2) 100,000 g pellet, LAE extract, *N. gonorrhoeae*, serotype A; (3) Fraction II, Sepharose 6B, *N. gonorrhoeae*, serotype A; (4) Fraction I, Sepharose 6B, *N. gonorrhoeae*, serotype A; (5) 100,000 g pellet, *N. meningitidis*, M986, serotype 2; (6) Lipopolysaccharide isolated from *N. gonorrhoeae*, serotype A, prepared as described in text. (B) Immunodiffusion in agar gel of LAE extracts of *Neisseria* sp. and *Escherichia coli*. Center well: antiserum prepared to 100,000 g pellet of LAE extracted *N. gonorrhoeae*, serotype B. Antigen wells: (1) Fraction I + II, Sepharose 6B, *E. coli*; (2) Fraction I, Sepharose 6B, *N. gonorrhoeae*, serotype B; (3) Fractions I + II, Sepharose 6B, *N. gonorrhoeae*, serotype A; (4) Fractions I + II, Sepharose 6B, *N. flava*; (5) Fractions I + II, Sepharose 6B, *N. gonorrhoeae*, serotype C; (6) Fractions I + II, Sepharose 6B, *N. gonorrhoeae*, serotype D.

the cross-reactive antigens present in all gonococcal serotypes. Lithium acetate extracts of *N. meningitidis*, group B, type 2, when analyzed by gel diffusion against eight antisera to eight different gonococcal serotypes, did not exhibit reactivity characteristic of the serotype antigen, but possessed cross-reactivity with Fraction II of these gonococcal extracts. Lipopolysaccharide extracted from several gonococcal serotypes did not react with either homologous or heterologous antisera. A TS or LAE extract of *E. coli* gave no reactivity with antisera prepared to the outer membrane of *N. gonorrhoeae*.

When freshly isolated colonial type T_1 colonies of gonococci were analyzed for serotype designation and compared to cells subcultured repeatedly to effect transformation to T_3 and T_4 forms, there was no detectable change in the major outer membrane protein, either with regard to its electrophoretic profile or its antigenic specificity. Thus, through repeated subculturing, the serospecific integrity of this protein is retained.

Cultures of gonococci which had not been selected for individual colonies after primary isolation on Thayer-Martin selective media often displayed mixed serotypes. However, when this heterogenous culture was replated and colonies selected at random, two or more serotypes present in the original isolate were separated. Table III tabulates the separation of five "mixed", nonselected primary cultures into their component serotypes.

Several instances of gonococcal transmission were investigated. When the gonococci isolated from partners of four heterosexual and three homosexual pairings were examined, the contact pairs were infected with the same serotype of gonococcus. In one instance involving two male homosexuals, the transmitted

TABLE III
Resolution of Gonococcal Isolates Having Heterogenous Serotypes into Separate Colonies Having a Single Serotype Designation

No. colonies selected	Serotype of heterogenous isolate	No. of colonies having serotype isolated after colony selection					
		A	B	C	F	G	N
21	A,C	14	—	7	—	—	—
15	A,F	12	—	—	3	—	—
17	C,G,N	—	—	2	—	8	7
18	A,B	2	16	—	—	—	—
16	F,N	—	—	—	13	—	3

serotype could be isolated from multiple sites, the pharynx, urethra, and rectum of the recipient. 16 gonococcal isolates from patients with disseminated gonococcal infection (gonococcaemia, gonococcal arthritis, salpingitis, gonococcal dermatitis) were examined and could be categorized into serotypes B, D, F, and H. On the basis of these preliminary data, there appears to be no relationship between a particular gonococcal serotype and invasiveness.

When the serological study was extended to include 203 strains of gonococci isolated in the New York City area, three more serotypes, H, N, and R, were discovered. These strains had major outer membrane proteins differing in molecular weight from the previously described eight serotypes. This brought the total number of distinct serotypes identified in the New York City area to 11 (Fig. 7). Using antisera prepared to the New York serotypes, strains of gonococci isolated from Taiwan, Denmark, Ethiopia, the Philippines, and Vietnam were typed. The result was a greater diversity than those isolated in New York (Fig. 8). Those isolates that could not be typed by using the 11 New York antisera were found to have unique outer membrane protein profiles, that is, a different subunit molecular weight for their major outer membrane protein (Table I, S, T, U, W, X). Antisera were prepared to those strains. Each gonococcal isolate was analyzed for serotype designation three times by immunodiffusion and by SDS-

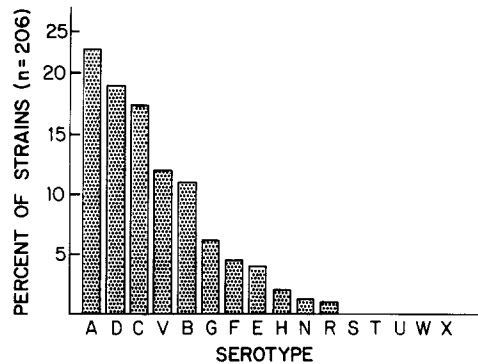


FIG. 7. Histogram of the distribution of serotypes of *N. gonorrhoeae* isolated from the New York City area. Bars represent the percentage of strains having a particular serotype in a total population of 206 isolates.

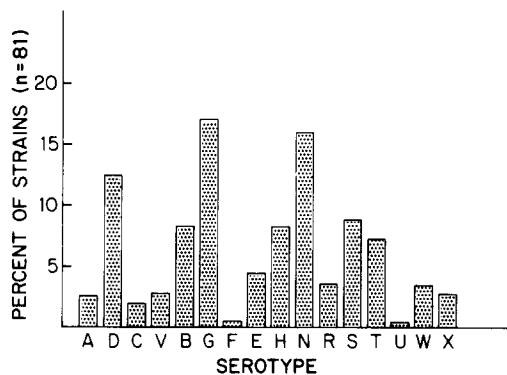


FIG. 8. Histogram of the distribution of serotypes of *N. gonorrhoeae* isolated from places other than from the New York City area. Strains of gonococci were isolated from Taiwan, Denmark, Ethiopia, Vietnam, and the Philippines. Bars represent the percentage of strains having a particular serotype in a total population of 81 isolates.

PAGE. In all instances, the serotype class, once established, did not change. Thus, during storage, the outer membrane protein complex does not undergo spontaneous antigenic variance as detected by our methods. These triplicate experiments done over a period of several months testify to the reproducibility of the procedure. Thus, to date, there are 16 distinct serotypes based on the antigenic specificity of the major outer membrane protein.

Discussion

The cell envelope of gram-negative bacteria is a structurally complex unit composed of three morphologically distinct layers. On the inner side of the rigid, peptidoglycan layer, is the cytoplasmic or inner membrane; on the outer side the outer or L-membrane. The latter contains substantial amounts of lipopolysaccharides, phospholipids, and proteins. The outer membrane of most gram-negative eubacteria is unique in that it has a relatively simple protein pattern (32, 33) with usually one or two protein species predominating. The outer membranes of *N. gonorrhoeae* (34, 46) and *N. meningitidis* (30, 47) are not exceptions. In our investigations of the outer membrane of the gonococcus, one protein, the major outer membrane protein, accounted for over 60% of the total membrane protein. This protein either represented a single molecular species or a mixture of proteins so similar in their subunit size that they could not be resolved by SDS-PAGE according to Weber and Osborn (42). It has been shown by Bragg and Hou (48) and Schnaitman (49, 50) that under certain conditions the major outer membrane protein of *E. coli* contains as many as three different polypeptides, each having a mol wt of 42,000 daltons. However, the major protein of the gonococcal outer membrane could not be resolved into more than one polypeptide by using either the Bragg-Hou method (48) or the Tris-glycine procedure of Maizel (43). Frasch and Gotschlich (30) demonstrated that the major outer membrane protein of *N. meningitidis*, group B, serotype 2, could not be separated into more than one polypeptide subunit, using the above electrophoretic methods. The nonpathogenic *Neisseria*, *N. sicca*, *N. flava*, and *N. lactamica*, also possessed a predominating protein in their outer membranes

which could not be fractionated by these procedures. This may reflect a fundamental difference in the structure of the major outer membrane protein of *Neisseria sp.* from that of *E. coli* (50), *Salmonella typhimurium* (32), and other gram-negative bacteria.

The isolation of this protein from gram-negative bacteria has entailed rather drastic procedures (31, 51). We used less severe methods of extraction such that we studied relatively undenatured protein. The method in our hands was to extract whole organisms with 200 mM lithium acetate buffer, pH 6.0 and 10 mM EDTA. This mild extraction method did not cause extensive lysis of the cells. Devoe and Gilchrist (52) have shown by electron microscopy that meningococci remain largely intact after a similar regime. The outer membrane is gradually peeled away in the form of small vesicles or blebs. These membrane units were shown to be representative of the outer membrane by chemical analysis and sucrose density centrifugation. They were of sufficient size to be excluded from a Sepharose 6B column which has an exclusion limit of 4×10^6 daltons for globular proteins. This membrane complex was composed entirely of the major outer membrane protein, a smaller covariant protein, and lipopolysaccharide. The difference in the quantity of this complex released from the outer membrane when intact cells were incubated in TS buffer, pH 8.0, and LAE buffer, pH 6.0, was striking.

The outer membrane of the gonococcus is the site of the serotype antigen complex. This was demonstrated by the almost identical SDS-PAGE protein profiles of isolated outer membranes and extracted serotype antigen complex. Because of this similarity, extracts of several gonococcal isolates were examined for similarities and dissimilarities in their protein profiles. Most noticeable was the variance in the electrophoretic mobility of the major outer membrane protein. 167 strains could be categorized into eight electrophoretically distinct classes. The classes were arbitrarily assigned a letter of the alphabet, A, B, C, D, E, F, G, and V. On the basis of quantity, apparent electrophoretic homogeneity, and variance within a population of gonococcal strains, the major outer membrane protein appeared to be a likely candidate for immunological study.

Antisera were raised in rabbits to each class of gonococcal isolates exhibiting different outer membrane protein profiles. When examined by immunodiffusion in agar-gels, those strains that had identical electrophoretic patterns also demonstrated immunological identity. Those strains that possessed a major protein of different subunit molecular size exhibited immunological nonidentity. A homologous reaction in agar gels gave a characteristic precipitin line concave to the antigen well. This is in keeping with the dynamics of the development of precipitin lines by double diffusion in agar-gels. The serotype antigen complex has a mol wt greater than 4×10^6 daltons, as evidenced by its exclusion from a Sepharose 6B column. When the serotype antigen complex was dissociated with either Triton X-100 or Brij-58, both nonionic detergents, and reacted with homologous antisera, the characteristic concave precipitin line was abolished. However, several precipitin lines were observed midway between the antigen and antibody wells resulting in observed cross-reactivity with heretofore distinct serotypes. This suggested that the polypeptide responsible for imparting serotype specificity is organized in the membrane complex such that the region

of the protein conferring type-specificity is exposed on the surface of the membrane, and that cross-reactive regions are most probably embedded in the membrane matrix. When whole cell vaccines were employed for the production of specific antisera, interpretation of precipitin lines was difficult, primarily due to the absence of strong positive reactions and the presence of minor nonspecific reactions. When isolated serotype antigens were used, the minor nonspecific reactions were abolished. Specific antisera could also be raised using isolated outer membranes. However, when these membranes were used as immunogens, it was important to monitor the antibody response. Antisera obtained 30 days after initial injection most often were suitable, but antisera obtained later in the course of immunization generally exhibited unwanted cross-reactions.

Gonococci can be divided into four colonial types by the presence of a degree of piliation (53, 54). Colonial types T₁ and T₂ are the virulent forms of gonococci (39, 55), whereas colonial types T₃ and T₄ are considered avirulent. Colony types T₁ and T₂ possess pili; colony types T₃ and T₄ do not (53, 54). When all four colony types of one strain were examined for serotype designation, no difference was detected between colonial types.

In using the serotyping system described above, it is important to take a random sampling of the gonococcal colonies isolated from an individual. This requirement was demonstrated by a number of isolates that upon primary analysis possessed multiple serotypes (Table III). By random picking of the original isolate, single serotypes were isolated. This indicated that a patient may harbor two or more gonococcal strains possessing different serotype antigens. Other workers using auxotyping of strains have come to the same conclusion (Personal Communication, Frank Young). This finding, the congruence of serotypes in contact pairs, and the fact that repeated analysis of 30 prototype strains always gave reproducible results suggest that the serotype specificity is a stable property of a gonococcal strain. Studies concerning the immune response to these antigens in infected human beings are in progress, and, if positive, would provide evidence that these antigens are expressed *in vivo*.

The isolation and identification of 16 serologically distinct strains of gonococci from a world-wide sample of strains demonstrates that the gonococcus as a species has evolved in such a manner as to permit the elaboration of a diverse antigenic profile. Five serotypes were isolated from Asiatic isolates which did not react with antisera produced to serotypes isolated in the New York City area. This may indicate that the number of potential serotypes may increase substantially with more sampling. This heterogeneity may provide an explanation for the observed lack of immunity after gonorrhoea. the relationship between antibiotic sensitivity and serotype diversity is an unanswered question, as is the relationship between serotype as defined in this communication and auxotype. These questions are the font of future investigations.

Summary

Neisseria gonorrhoeae has been subdivided into several classes of serologically distinct groups. The serotyping system is based upon the antigenic specificity of a protein serotype antigen. This protein is the major polypeptide of the outer membrane of the gonococcus and accounts for over 60% of that membrane's

total protein. The serotype antigen complex was isolated by mild extraction of intact organisms in 200 mM lithium acetate buffer, pH 6.0 with 10 mM EDTA for 2 h at 45°C. The extract was fractionated on Sepharose 6B and partially purified by precipitation at pH 4.2 by addition of 10% (vol/vol) acetic acid. Each serotype antigen has a unique subunit molecular size as determined by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Preliminary typing of a gonococcal strain may be performed by comparative SDS-PAGE. To date, 16 different serotypes, representing a diverse distribution, have been isolated.

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