PURIFICATION AND PARTIAL CHARACTERIZATION OF THE OPACITY-ASSOCIATED PROTEINS OF *NEISSERIA GONORRHOEAE*

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Differences in colonial morphology of gonococci grown on solid medium have been observed for several years (1). A classification scheme based on these differences was first introduced by Kellogg (2, 3). It was later determined that the basis of the Kellogg typing system was associated with the expression or lack of expression of pili by the bacteria (4). Swanson (5) later modified this scheme based on the transmission of reflected light through the gonococcal colonies. Bacterial colonies were thus described as either transparent or opaque. Swanson (6) related this difference in colonial morphology with the appearance of one or sometimes two additional proteins, the opacity proteins, in the outer membrane of gonococci displaying the opaque phenotype, which were not present in isogenic transparent clones (7).

Interest in these gonococcal opacity variants was further stimulated when James and Swanson (8), and later Draper et al. (9), showed that the isolation of a particular colony phenotype was related to the female menstrual cycle and/or the anatomical location of isolation. Thus, gonococci expressing a transparent phenotype were more likely to be isolated from cervical cultures of a patient during her menses and an opaque phenotype during ovulation.

Opaque gonococci, when viewed by transmission electron microscopy, appear to have numerous intercellular adhesions that cause the organisms to aggregate in large clumps (5). Such adhesions are not observed in transparent organisms. This aggregation is temporarily lost upon trypsinization of the opaque gonococci (5). Upon investigation of the effect of trypsin and other enzymes on the opaque protein, it was shown that these enzyme readily cleave Proteins II, leaving only a small peptide associated with the outer membrane (10).

In order to more clearly define the role and function of opacity-associated proteins, a method was devised to isolate and purify these proteins on a large scale. We will also describe some of the biochemical and immunochemical characteristics of these proteins.

Materials and Methods

Bacteria and Culture Conditions. Nonpiliated opaque phenotypes of gonococci were maintained by single colony transfers every 18–24 h on solid typing media (5), and

This work was supported in part by Public Health Service grants AI 10615 and AI 18637 from the National Institutes of Health and by a grant from The Rockefeller University Biomedical Fund S07RR07065.

 J. Exp. Med. © The Rockefeller University Press · 0022-1007/84/02/0452/11 $1.00
Volume 159 February 1984 452–462

incubated at 36°C in candle extinction jars. The bacteria were verified as *N. gonorrhoeae* by Gram's staining, and oxidase and fermentation reactions. Although several strains have been studied, the majority of the work described here was performed using strain R10.

The growing of the gonococci in a 60 L fermentor was performed as previously described (11).

Purification. All purification steps were carried out at room temperature unless stated otherwise. The harvested bacteria were slowly resuspended in an equal volume of 1.0 M sodium acetate, pH 4.0 containing 1 mM 2,3-dimercaptopropanol. To this suspension was added 9 volumes of 5% (wt/vol) *N*-tetradecyl-*N*,*N*-dimethyl-3-ammonia-1-propanesulfonate (Z 3,14)(Calbiochem-Behring Corp., La Jolla, CA) in 0.5 M $CaCl_2$ and stirred for 1 h. Absolute ethanol was added slowly to bring the concentration to 20% (vol/vol). The precipitate, which included most of the nucleic acids, was removed by centrifugation at 17,000 *g* for 10 min. The concentration of ethanol was then increased to 80% (vol/vol) and the resultant precipitate recovered by centrifugation. This precipitate, which contained the porin protein and the proteins II was resuspended in 50 mM Tris-HCl, pH 8.0, 10 mM EDTA and 0.05% Z 3,14. This mixture was stirred for 1 h, centrifuged at 12,000 *g* for 10 min, and the soluble material retained.

This solution was applied to two columns (2.6 × 60 cm) linked in tandem, one packed with DEAE-Sepharose 6B-CL (Pharmacia Fine Chemicals, Piscataway, NJ) and the second packed with CM-Sepharose (Pharmacia Fine Chemicals) both equilibrated with 50 mM Tris-HCl pH 8.0 with 10 mM EDTA and 0.05% Z 3,14. The sample was applied to the DEAE-Sepharose column and the two columns were washed until the 280 nm absorbance fell to baseline. The two columns were then separated, a linear NaCl gradient between 0.0 to 0.5 M in a total volume of 700 ml applied to the CM-Sepharose column and the protein elution pattern monitored by 280 nm absorption and SDS-PAGE analysis. Fractions containing the opacity-associated protein were pooled, the proteins precipitated by the addition of ethanol to a final concentration of 80% (vol/vol), and the precipitate collected by centrifugation. The precipitate was resuspended in 15 ml of the Tris-EDTA 5% Z 3,14 buffer and applied to a column (2.6 × 170 cm) of Sephacryl S-200 (Pharmacia Fine Chemicals). The elution buffer consisted of 10 mM Tris-HCl, 200 mM NaCl, 10 mM EDTA, 0.05% Z 3,14 at pH 8.0 and the flow rate was 10 ml/h. Fractions of 7.5 ml were collected, monitored for absorption at 280 nm, and analyzed by SDS-PAGE.

Amino Acid and Carbohydrate Analysis. Gas-liquid chromatography (GLC)[1] methodology described by Clamp et al. (12), was used to measure the carbohydrate content of the purified protein. The exact procedure used has been described previously (11). The amino acid composition was determined using a Durrum Model D500 amino acid analyzer (Beckman Instruments, Palo Alto, CA). Briefly, samples were prepared by lyophilizing 5 to 6 nmol of protein and hydrolyzing with 50 μl of methanesulfonic acid in evacuated sealed vials at 115°C for 20 h (13). The pH of the sample was adjusted to 2.2 and the sample applied to the analyzer.

Determination of Amino-terminal Amino Acids. The amino terminus was determined using the dansyl chloride labeling method of Gray (14). After reaction with dansyl chloride, the protein was hydrolyzed by the addition of 50 μl of redistilled constant-boiling HCl, the tube evacuated, and sealed. Hydrolysate was dried under a nitrogen stream, redissolved in 10 μl of 50% pyridine, and applied to polyamide sheets for two-dimensional thin layer chromatography as described by Woods and Wang (15).

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS–PAGE). The modifications and sample preparations of the Laemmli SDS-PAGE method (16) using a discontinuous buffer system has been described in detail elsewhere (17).

Isoelectric Focusing. Two analytical methods were used to determine the isoelectric point of Protein II. One method described by Ames (18) determines the isoelectric point in denaturing conditions and was performed using polyacrylamide as the supporting material. The samples were prepared in boiling 2% SDS and 8 M urea and applied to the acidic end, as described previously (17). The isoelectric point of Protein II in non-

[1] *Abbreviations used in this paper:* GLC, gas liquid chromatography; LPS, lipopolysaccharide; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

denaturing conditions was determined using the methods of Rosen et al. (19). The support material was 0.8% Isogel (Marine Colloids Div., Rockland, ME) to which ampholines and Z 3,14 had been added. Purified protein II in 1% glycine and 0.05% Z 3,14 was applied directly to the agarose surface using small pieces of Whatman 3MM filter paper (Whatman Inc., Clifton, NJ).

Immunological Methods. Antisera to whole organisms were produced as described by Johnston et al. (20). Antisera to purified protein II were produced by injecting rabbits subscapularly, first with 0.1 mg of purified protein in Freund's complete adjuvant (Difco Laboratories, Detroit, MI), then after 3 wk the rabbits were reinjected in the same manner but with 0.1 mg protein in Freund's incomplete adjuvant (Difco Laboratories). Before each bleeding, the rabbits were injected intravenously every other day for 2 wk with the same dose of protein II in saline and then bled after a 2-wk waiting period. The sera were monitored for anti-protein II antibodies by the ELISA technique. To sensitize the ELISA plate (Immunolon II; Dynatech Labs Inc., Alexandria, VA) purified protein II was diluted to 2 μg/ml in 0.1 M Tris-HCl, pH 9.8, 100 μl was added to each well, and incubated at room temperature overnight. The plates were washed six times with 0.05% Brij 35 in 10 mM Na Acetate, 0.2 M NaCl, pH 6.8 and the antisera diluted in 0.05% Brij 35 in PBS and added to the plates. After a 4-h incubation, the plates were washed as before and the alkaline phosphatase-goat antirabbit conjugate (Tago, Inc., Burlingame, CA) was added in 0.05% Brij PBS solution. After 4 h, the plates were washed, the substrate *p*-nitrophenyl phosphate (Sigma Chemical Co., St. Louis, MO) in 1 M Tris-HCl, pH 9.6 (1 mg/ml) added, and the product of the alkaline phosphatase reaction determined after 1 h at 37°C using a Titertek Multiskan (Flow Laboratories, McLean, VA) set at 405 nm. The reciprocal of the dilution giving a reading of 1.00 was used to compare antisera.

Results

Purification

$CaCl_2$-zwittergent Z 3,14 extraction. When 5% Z 3,14 in 0.5 M $CaCl_2$ was added to a suspension of gonococci, it solubilized several of the outer membrane proteins and released much of the nucleic acids. By the addition of ethanol to 20%, most of the nucleic acids were precipitated. This extraction left protein II in the supernate. It was found that by varying the pH of the solubilization solution, specific proteins could be extracted. At a pH of 4.0, all of the proteins II as well as the porin proteins (protein I) were released and protein III, a protein closely associated with protein I, remained in the ethanol-precipitable fraction, as shown in Fig. 1. To prepare the proteins for ion exchange chromatography they were precipitated with ethanol and resuspended in Tris-HCl-Z 3,14 buffer.

Ion-exchange chromatography. The isoelectric points of proteins I and the opacity-associated proteins from several different strains were determined and found to be between 5.0 and 7.5 for protein I and 9.0 and 10.0 for opacity-associated proteins. We found that by chromatographing at pH 8 the mixture of the two proteins on two ion exchange columns linked in tandem, that the opacity-associated proteins could be separated from protein I. The proteins were then eluted from the CM-Sepharose column with a NaCl gradient in one or two peaks (depending on the number of proteins II expressed by the particular strain) between 15.0 and 18.0 mMhos (Fig. 2).

Molecular sieve chromatography. Protein II eluted from a Sephacryl S-200 column in a single peak with a K_{av} of 0.167 (Fig. 3). This would suggest that the protein was migrating as a monomer of 30,000 mol wt once the micellar molecular weight of the Zwittergent was taken into account. The protein profile

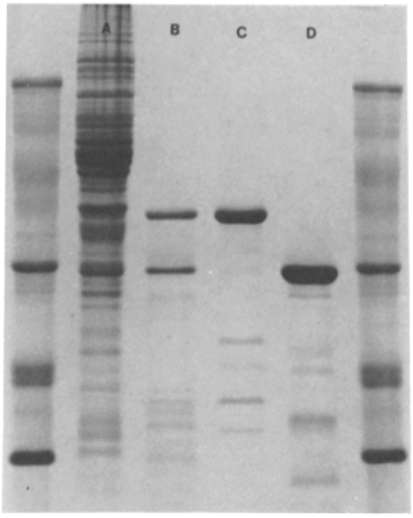

FIGURE 1. SDS-PAGE protein profiles of a $CaCl_2$-Zwittergent extraction of gonococci producing both protein I and II. Lane *A* represents the starting material and is a whole cell lysate of opaque organisms. Lane *B* shows the proteins that remain soluble at pH 4.0 with 20% ethanol. Lane *C* represents those proteins that are retained on a DEAE-Sepharose column at pH 8.0 and eluted with NaCl. Lane *D* shows those proteins that are eluted from a CM-Sepharose column with the same conditions. The molecular weight standards in the outermost lanes are human transferrin (80,000), carbonic anhydrase (30,000), soybean trypsin inhibitor (21,500), and cytochrome c (12,500). Note that both protein I and II are extracted with the $CaCl_2$-Zwittergent and remain soluble in the 20% ethanol. These two proteins and their breakdown products represent the majority of the proteins observed in this fraction.

on SDS-PAGE showed that this peak contained pure protein II (Fig. 4). The typical yield from 100 g (wet weight) of bacteria was 100 mg.

Immunological properties. As seen in Table I, all rabbits injected with phenotypically opaque bacteria or with purified proteins II produced antibodies reacting to protein II. When the sera from each rabbit were reacted with proteins II extracted from different gonococcal strains, a spectrum of reactivities was observed. The individual serum from rabbits injected with intact organisms reacted in the ELISA in one of three distinct ways: (*a*) the serum reacted best with the protein II from the homologous strain, such as sera 545 and 541; (*b*) the serum reacted better with two proteins II than a third, such as sera 543, 542, and 481; and (*c*) the serum reacted with all three proteins II tested equally, such as sera 538 and 537. A comparison of the antisera raised using purified proteins II gave a similar spectrum.

Isoelectric focusing. When isoelectric focusing was performed using the methods of Ames (18) under denaturing conditions, the purified protein II migrated into the basic buffer and was not retained within the gel matrix. Even when additional basic ampholines were added to this system, we were unable to increase the basic end of this gel system above a pH of ~8.5. We, therefore, switched to an agarose system that allowed us to focus up to a pH of 10.0. In this system with the addition of the Z 3,14, the proteins II focused between 9.0 and 10.0 with the majority focusing at a pH of 9.6, as shown in Fig. 5.

Amino acid and chemical analyses. Protein II lacked any associated carbohydrate

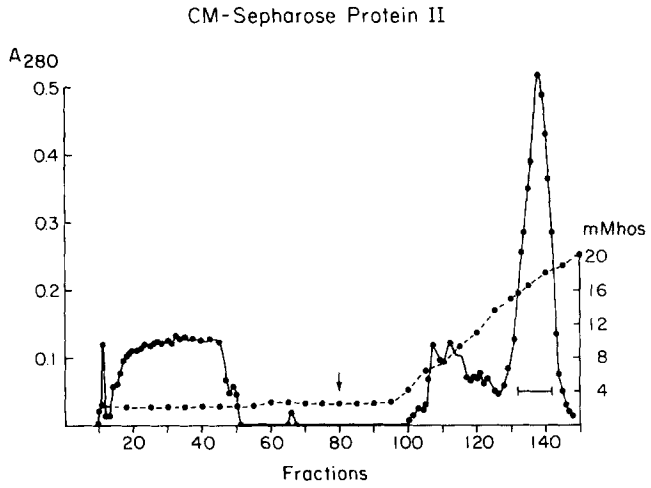

FIGURE 2. Elution profile of the opacity-associated protein from a CM-Sepharose column (2.6 × 60 cm). The sample was applied in a buffer consisting of 50 mM Tris-HCl, pH 8.0, 10 mM EDTA, and 5% Z 3,14. The column was washed with the same buffer with 0.05% Z 3,14 until the 280 nm absorbance fell to zero. The column was eluted with a NaCl gradient between 0.0 to 0.5 M with a total volume of 700 ml. The elution was monitored by absorbance at 280 nm (solid line), conductivity (dashed line), and SDS-PAGE analysis. Protein II eluted from the column between 15 and 18 mMho. The fractions containing protein II were collected and pooled as shown by the bar.

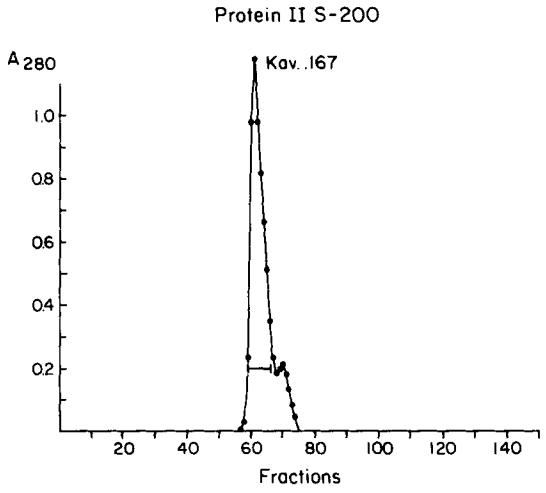

FIGURE 3. Protein II eluted from a Sephacryl S-200 column (2.6 × 170 cm) with a K_{av} of 0.167. The elution buffer consisted of 0.1 M Tris-HCl, pH 8.0, 0.2 M NaCl, and 0.05% Z 3,14. The elution was monitored by absorbance at 280 nm (solid line) and SDS-PAGE analysis. Fractions of 7.5 ml were collected and those containing protein II were pooled as indicated.

as determined by GLC analysis. In comparison with protein I from the same strain, protein II had by amino analysis (*a*) more aliphatic residues, (*b*) fewer aromatic residues, (*c*) only one proline, (*d*) fewer dicarboxylic amino acids, and (*e*) more basic amino acids with a large number of arginyl residues (Table II).

Amino-terminal determination. By the standard manual amino-terminal deter-

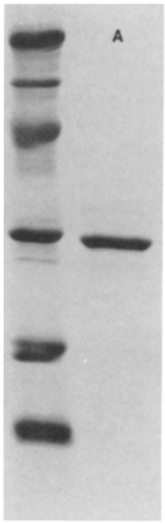

FIGURE 4. SDS-PAGE analysis of the purified protein II from strain R10 (lane *A*), and molecular weight standards: human transferrin (80,000), catalase (60,000), ovalbumin (45,000), carbonic anhydrase (30,000), soy bean trypsin inhibitor (21,500), and cytochrome c (12,500).

TABLE I
Immunological Reactivities of Protein II Antisera

Antigen used for immunization	Serum no.	Strain origin of Protein II used in ELISA plates		
		F62	MS11	R10
Whole cells strain (phenotype)				
F62 (p + op)	545	16.0*	3.7	4.6
F62 (p + op)	543	23.4	13.5	22.2
MS11 (p + op)	538	61.5	19.7	14.8
MS11 (p − op)	537	315.1	275.7	236.3
R10 (p + op)	541	2.0	1.4	11.1
R10 (p + op)	542	2.3	0.8	9.2
R10 (p − op)	481	103.4	23.4	167.4
Purified proteins from strain				
F62	517	4.0	1.6	2.0
F62	522	2.0	0.3	0.5
R10	525	3.7	0.9	3.9
R10	638	—	—	2.5
R10	639	0.8	—	6.5
R10	858	5.9	4.9	4.0

* The reciprocal of the antiserum dilution × 10^{-2} that gave an ELISA reading of 1.00 after 1 h at 37°C.

mination, the results were negative. This suggested that the amino-terminus of protein II was blocked or unavailable. This was also confirmed by introducing the protein dissolved in 50% (vol/vol) acetic acid into a model 890B sequenator (Beckman Instruments, Fullerton, CA). However, when proteins II were first precipitated with 10% trichloroacetic acid, the precipitate washed with ethanol

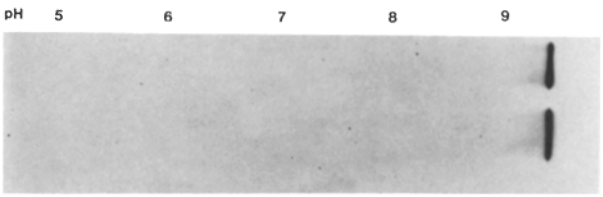

FIGURE 5. Isoelectric focusing analysis performed in agarose of purified protein II from strain R10. The pH gradient in measured by cutting the gel in 0.5-cm sections, soaking the sections in distilled water, and determining the pH. The protein in the top lane was applied on the left, and that in the lower lane, on the right.

TABLE II

Amino acids	Protein I		Protein II
Neutral	206*	(0.67)†	163 (0.62)
Aliphatic	162	(0.53)	147 (0.56)
glycine	42		32
alanine	27		26
valine	27		29
leucine	18		15
isoleucine	6		11
serine	27		21
threonine	15		13
Aromatic	27	(0.09)	10 (0.04)
phenylalanine	12		5
tyrosine	15		5
tryptophan	—		—
Sulfur-containing	5	(0.02)	5 (0.02)
half cystine	1		—
methionine	4		5
Amino acids/proline	12	(0.04)	1 (0.003)
Dicarboxylic amino acids	69	(0.23)	58 (0.22)
Aspartic/asparagine	33		42
Glutamic/glutamine	36		16
Basic amino acids	30	(0.10)	41 (0.16)
Histidine	6		4
Arginine	6		22
Lysine	18		15
Total	305		262

* Number of moles of residue per mole of protein.
‡ Fraction of total number of residues represented in each class.

and acetone, and applied into the sequenator in 50% acetic acid, the amino-terminal was found to be as shown in Fig. 6.

Discussion

The majority of the protein located in the outer membrane of the gonococcus consists of proteins I and II. The treatment of gonococci with calcium and detergent releases these two proteins completely and the majority of the other proteins that one sees in whole cell lysates of this organism remains precipitated

THE AMINO-TERMINAL AMINO ACID SEQUENCES OF PROTEIN II

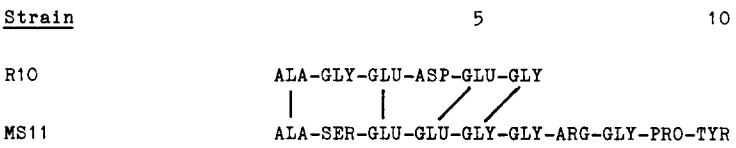

FIGURE 6. The amino-terminal sequence of proteins II of *N. gonorrhoeae* strain R10 and MS11. Solid lines represent identical residues. Note that with the exception of the GLY for the SER substitution in the second residue and the deletion of ASP residue in the MS11 protein II, the sequences are identical.

in the 20% ethanol fraction. The effectiveness of high concentrations of calcium in this method may be because: (*a*) increased calcium may cause lipolysaccharide (LPS) to aggregate into large clusters and increase the rigidity of the outer membrane (unpublished results) (21–24); and (*b*) with increased membrane rigidity it has been shown that larger portions of integral membrane proteins are exposed at the surface (25). We hypothesize that as the concentration of calcium is increased, the LPS associates into large aggregates and decreases its association with the membrane proteins. This would then reveal hydrophobic sites located on these proteins. Thus, with the addition of the zwitterionic detergent, these hydrophobic sites are covered with the detergent and the proteins are released from the membrane.

All of the opacity-associated proteins that we have observed have had an isoelectric point higher than pH 9.0. Proteins I usually have an isoelectric point below a pH of 8.0. Therefore, the difference in isoelectric points allowed us to separate these two proteins on tandem columns of different charge, as seen in Fig. 1. Proteins I are either eluted in the flow through of both columns or maintained on the DEAE-Sepharose. The opacity-associated proteins on the other hand are maintained on the CM-Sepharose and eluted with NaCl. The basic nature of these proteins was also evident in the isoelectric focusing of the purified proteins and in their amino acid analysis. The large amounts of arginine in these molecules may also be related to their high susceptibility to trypsin, which had been observed previously (10).

The proteins II isolated with this method appeared to migrate as monomers in the molecular sieving columns. This is in contrast with proteins I, which are trimers in solution. No co-migrating proteins were observed in the SDS-PAGE analysis of the fractions from gel filtration chromatography. These observations would suggest that the inter-membrane adhesions that are observed with bacteria expressing the opacity-associated proteins are not due to protein II–protein II interaction or protein I–protein II interactions. If such interactions were occurring, they would be detected by either the lowering of the K_{av} at which the opacity proteins eluted from the molecular sieve chromatography and/or the SDS-PAGE analysis of the column fractions would show co-migrating proteins other than protein II. We are presently exploring other moieties of the gonococcal outer membrane for the ligand that may be important for this adhesive property.

The immunological data on protein II showed that antisera raised in rabbits

by injecting either whole cells or purified proteins yielded three types of reactions. One group of rabbits produced antibodies that were more reactive with the homologous protein II than with the other two heterologous proteins II. A second group of antisera showed a limited cross-reaction, reacting with two proteins II but not the third. The third group reacted almost equally with the three different proteins II. These three types of reactions were observed both when intact gonococci and when purified proteins II were used as immunogens. It has been suggested by Swanson and Barrera (26) that when whole gonococcal cells are used as immunogens, the antibodies raised are rather specific in nature and do not cross-react with proteins II of other strains or with other proteins II of the same strain. Furthermore, they suggest, that proteins II, isolated by the methods described here or by isolating bands from SDS-PAGE gels, when used for immunization, gave rise to cross-reactive antibodies (26). Our observations suggest that the cross-reactions of sera may relate more to the immunization schedule used in these studies and less to the physical state of the proteins II. Nevertheless, this would suggest the existence of common domains on all proteins II and that these common domains are hidden by other components of the gonococcal outer membrane or by the conformation of the molecule as it exists within this membrane. Thus, this molecule, much like gonococcal pili and protein I, would appear to have a common domain that is masked or hidden and a variable region that is extensively exposed on the surface of the bacterium. That there are common peptides has been previously documented (27). If protein II is similar to pili, the functional portions of the molecule might be located within these common areas (28). We are now exploring this possibility.

Summary

Gonococci, grown on agar, frequently give rise to opaque colonies. This opacity phenotype is associated with the presence of one or more outer membrane proteins of ~28,000 mol weight. These proteins are included within a class of proteins named proteins II. A method is described to isolate and purify the opacity-associated proteins from *Neisseria gonorrhoeae.* This method uses high concentrations of calcium and a zwitterionic detergent at pH 4.0. Under these conditions proteins II are readily solubilized from the outer membrane. Further purification is achieved by ion exchange and molecular sieve chromatography in the presence of the zwitterionic detergent. The opacity-associated proteins are very basic with isoelectric points varying between 9.0 to 10.0. Further evidence for their basic nature is their behavior on ion exchange chromatography and their amino acid composition.

Received for publication 28 September 1983.

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