INFECTION AND IMMUNITY, July 1981, p. 212-222
0019-9567/81/070212-11$02.00/0

Vol. 33, No. 1

Effects of Proteolytic Enzymes on the Outer Membrane Proteins of *Neisseria gonorrhoeae*

MILAN S. BLAKE,[1]* EMIL C. GOTSCHLICH,[1] AND JOHN SWANSON[2]

Rockefeller University, New York, New York 10021[1] and Laboratory of Microbial Structure and Function, National Institute of Allergy and Infectious Diseases, Rocky Mountain Laboratory, Hamilton, Montana 59840[2]

Received 26 January 1981/Accepted 9 April 1981

Proteolytic enzymes inhibit the growth of some strains and opacity variants of *Neisseria gonorrhoeae*. To understand the inhibitory effects of these enzymes, we examined several strains to determine the actions of proteases on the three predominant proteins in gonococcal outer membranes, namely, the major outer membrane protein (protein I), the sometimes-expressed opaque protein (protein II), and protein III. In a comparison of the protein I species expressed by different strains, we observed a pattern based on subunit molecular weight and susceptibility to enzymatic degradation. Protein I species having molecular weights of 34,000 were more susceptible to proteolysis, whereas protein I species having molecular weights of 33,000 were less susceptible, and protein I species having molecular weights of 32,000 were resistant. This pattern was observed both in intact cells and in purified outer membranes. All of the enzymes degraded protein II, but this susceptibility appeared to be influenced in part by the species of protein I present. Protein III was resistant to all of the proteolytic enzymes tested. Based on the resulting fragments from each proteolytic cleavage of proteins I and II and their membrane associations, we suggest how these proteins may be arranged in intact membranes. Our data suggested the presence of an endogenous gonococcal enzyme. This enzyme appeared to degrade proteins I and II into fragments resembling the fragments resulting from the action of chymotrypsin.

Many of the variations observed in the colonial morphology of gonococci grown on solid media can be correlated with differences in surface components. Kellogg et al. described a classification of gonococci based on colonial morphology and showed that gonococci exhibiting a particular colonial phenotype could be maintained by selective transfer (11). Kellogg et al. also correlated increased virulence in humans with particular colony forms, which they called type 1-2 gonococcal colonies (10). Recently, Swanson suggested that the original typing system of Kellogg et al. should be modified to include two new colony phenotypes based on the opacity of colonies (18). In comparisons of these two colony phenotypes, it has been shown that gonococci expressing the opaque colonial phenotype (O^+) (i) typically are isolated from the urethrae of males and from the cervices of women near ovulation (7), (ii) grow on solid media in colonies which are opaque to transmitted light (7, 18), (iii) are less virulent in the chicken embryo model (16), (iv) express an extra dominant heat-modifiable protein in the outer membrane (protein II) (13, 18), and (v) show inhibition of growth when exposed to trypsin (7). In contrast, gonococci of the transparent colonial phenotype (O^-) (i) typically are isolated from cervices of women near menstruation and from fallopian tubes at all times of the menstrual cycle (4, 7), (ii) grow in colonies which are transparent (18), (iii) are more virulent in the chicken embryo model (16), (iv) lack the heat-modifiable protein (13, 18), and (v) are less sensitive to growth inhibition by trypsin (7). When serial endocervical cultures were obtained, the infecting strain changed from O^+ to O^- corresponding to the menstrual cycle of the patient (7). The O^- phenotype can be selected in vitro by exposing the gonococci to trypsin (7). It was this last phenomenon which led us to study the effects of several proteolytic enzymes not only for their ability to inhibit the growth of gonococci but also for their action on proteins of the outer membrane.

Intact outer membranes of gonococci can be isolated relatively easily because of the tendency of gonococci to shed portions of this membrane in the form of small vesicles or blebs (8). The proteins of this membrane can be grouped into three families which are particularly prominent. We examined the sodium dodecyl sulfate (SDS)-

polyacrylamide gel electrophoresis (PAGE) protein profiles of both intact cells and purified outer membranes for changes in these proteins after proteolysis with several enzymes. We found a clear correlation between the susceptibility of the membrane proteins to enzymatic attack in situ and the inhibitory effect of the enzymes on the growth of the organisms on solid media. The first and usually most abundant group of proteins includes the major or principal outer membrane proteins (protein I). This group of proteins is analogous to protein I of *Escherichia coli*, and we have found that the members of this group are porins (F. Greco, M. S. Blake, E. C. Gotschlich, and A. Mauro, Fed. Proc. **39**:1813, 1980). They also constitute the major serotyping antigens described by Johnston et al. (9, 15). Using the peptide mapping techniques of Elder et al. (5), Swanson subdivided these proteins into three distinct species, one migrating at a molecular weight of 34,000 (34K) on SDS-PAGE, one migrating at 33K, and one migrating at 32K (19). We found additional evidence for distinguishing these three subgroups of protein I. In intact organisms and purified membranes, the 32K protein I species were resistant to degradation by all of the enzymes which we used. However, the 34K protein I species were cleaved readily by the enzymes tested, and the 33K protein I species were intermediate in their susceptibility to enzymatic attack. The proteolysis of the 34K and 33K protein I species by alpha-chymotrypsin produced two fragments, both of which remained tightly bound to the membrane after centrifugation.

A second group of prominent proteins is expressed only in O^+ organisms, and these are the so-called opaque or heat-modifiable proteins (protein II). These proteins are very susceptible to proteolysis and are not visible on SDS-PAGE after trypsin treatment of O^+ organisms (18). Compared with the other two protein groups, protein II seems to be the protein most available for enzymatic degradation. We found that after proteolytic digestion of protein II, only one fragment remained within the membrane. The presence of protein II enhanced the proteolytic action of the enzymes tested on both degradable protein I subgroups. The reverse seemed to be true also; namely, the more susceptible the protein I, the more readily protein II was cleaved. A third protein, protein III, occurs in the outer membranes of all gonococci. McDade and Johnston showed that this protein does not behave as a heat-modifiable protein but does undergo a change in mobility on SDS-PAGE gels after treatment with 2-mercaptoethanol (15). Protein III seemed to be unaffected by the enzymes tested.

MATERIALS AND METHODS

Chemical methods and reagents. Protein concentrations were determined by the Coomassie blue method (17). The enzymes used and their sources were as follows: alpha-chymotrypsin (EC 3.4.21.1), Sigma Chemical Co., St. Louis, Mo., and Worthington Diagnostics, Freehold, N.J.; protease from *Streptococcus griseus*, Sigma Chemical Co. and Calbiochem, La Jolla, Calif.; and tolylsulfonyl phenylalanyl chloromethyl ketone (TPCK)-trypsin (EC 3.4.21.4), Worthington Diagnostics.

Bacteria. More than 12 gonococcal strains were examined by one or more of the techniques described below. The strains specifically discussed in this report were as follows: strain F62, maintained in our laboratories for several years; strain MS11/020780, a spontaneous mutant from strain MS11; strain R10, obtained from the Department of Microbiology, New York Hospital-Cornell Medical School, New York, N.Y.; and strains 120176-2 and 10677-2, obtained from the Venereal Disease Clinic, Salt Lake City, Utah. All strains were identified as *Neisseria gonorrhoeae* by Gram staining and by oxidase and fermentation reactions. Nonpiliated O^- and O^+ phenotypes were used in this study and were maintained by single-colony passages every 18 to 24 h on solid typing media, as described by Swanson (18). To maintain isogenic clones of each opacity variant, a colony of the opposite opacity phenotype was selected and subcultured every 10 to 15 days; e.g., an O^- colony was selected from a plate of colonies expressing the O^+ colonial phenotype.

Gonococcal growth inhibition by proteolytic enzymes. The effects of proteolytic enzymes on the growth of gonococci were determined as described by James and Swanson (7). These experiments were performed in duplicate by using the same batch of agar medium on the same day. A single colony of the desired phenotype was removed from each culture plate with a small piece of filter paper, which was agitated vigorously in proteose peptone saline or phosphate-buffered saline with a Vortex mixer. A portion of this suspension (100 μl) was spread onto a plate of solid medium with a bent glass rod. After incubation at 36°C for 2 h, a disk was placed on the plate and saturated with the appropriate enzyme (10 mg/ml) in buffer. After 24 h of incubation, the zone of growth inhibition was measured with a micrometer eyepiece in three different directions, and the average value was recorded.

Bactericidal actions of proteases. Gonococci propagated on solid medium for 21 to 24 h were suspended in 0.046 M tris(hydroxymethyl)aminomethane (Tris) hydrochloride containing 0.0115 M $CaCl_2$ (Tris-$CaCl_2$ buffer) at pH 8.2 to an optical density at 540 nm of 0.9. A 1.5-ml portion of this suspension was centrifuged, and the resulting pellet was suspended in 500 μl of the same buffer. A 200-μl sample of this suspension was mixed with an equal volume of buffer or of buffer containing alpha-chymotrypsin (40 μg/ml), and the mixture was incubated at room temperature. Portions (25 μl) were removed at zero time and at 15 and 30 min; these portions were diluted and plated for estimating colony-forming units.

Enzyme treatment of intact gonococci. Gonococci grown for 22 to 24 h were swabbed from solid

medium and suspended in Tris-$CaCl_2$ buffer or phosphate-buffered saline to an optical density at 540 nm of 0.96, and 1.5 ml of this suspension was centrifuged in a Microfuge (Beckman Instruments, Inc., Palo Alto, Calif.). The resulting pellet was suspended in 500 μl of Tris-$CaCl_2$ buffer, and 75-μl samples were dispensed into small (170-μl) Microfuge tubes. To each sample an equal volume of either buffer or proteolytic enzyme was added. After incubation for 15 min at room temperature, the cells were pelleted by centrifugation and solubilized in 25 μl of solubilizing solution containing SDS. Each tube was boiled immediately in a water bath for a minimum of 5 min.

^{125}I labeling. Gonococci to be labeled were grown, suspended, and pelleted as described above, and then they were suspended in 50 μl of buffer (phosphate-buffered saline, HEPES [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid]-saline, or Tris-$CaCl_2$). Each suspension was transferred to a glass vial previously coated with Iodogen (Pierce Chemical Co., Rockford, Ill.) by allowing 10 μl of a 1-mg/ml chloroform solution to evaporate (14); 10 μl of ^{125}iodine (diluted to approximately 1 μCi/μl with 10^{-6} M KI; New England Nuclear Corp., Boston, Mass.) was added to the bacterial suspension, and the mixture was incubated at room temperature for 5 to 10 min. Unbound ^{125}I was removed from the labeled bacteria by two cycles of centrifugation and resuspension in 500 μl of Tris-$CaCl_2$. The resulting pellet was suspended in 500 μl of Tris-$CaCl_2$ and used for enzymatic digestions, as described above.

SDS-PAGE. Electrophoresis was carried out in gels by using the Laemmli buffer system (12) at polyacrylamide concentrations ranging from 12.5 to 18%. The procedures used have been described previously (19). Autoradiography of the gels was performed by placing wet gels covered by Saran Wrap on radiographic film (Kodak X-OMAT; Eastman Kodak Co., Rochester, N.Y.) for 1 to 3 days at room temperature.

Peptide mapping. ^{125}Iodine peptide maps of protein I and its fragments were produced as described by Swanson (19), using the techniques of Elder et al. (5).

Purification of outer membranes. The liquid medium used consisted of a dialysate prepared as follows. To prepare 20 liters of medium, 225 g of proteose peptone (Difco Laboratories, Detroit, Mich.), 80 g of K_2HPO_4, 20 g of KH_2PO_4, and 100 g of NaCl were dissolved in water, and the volume was adjusted to 4,000 ml. This 5× concentrate was filtered by using diatomaceous earth (grade I; Sigma Chemical Co.) and concentrated by using an Amicon DC2 fiber filter concentrator with a PM-10 filter (Amicon Corp., Lexington, Mass.). The diffusate was collected and stored frozen at −76°C until it was used. The proteose peptone dialysate was diluted with 4 volumes of water, and 1,500-ml volumes were placed in 2,800-ml triple-baffled Fernbach flasks (Bellco Glass Inc., Vineland, N.J.) and autoclaved. To each flask 15 ml of a solution identical to IsoVitaleX (BBL Microbiology Systems, Cockeysville, Baltimore Md.) was added. Approximately 10^{10} bacterial cells from one petri dish (15 by 100 mm) were inoculated into each of the Fernbach flasks, and these cultures were incubated at 36.5°C while shaking at 100 rpm in a Brunswick rotary shaking incubator (New Brunswick Scientific Co., New Brunswick, N.J.) for 7 h or until late log phase. Typically, six flasks were used for each variant, and the two variants were grown and their outer membranes were extracted on the same day.

The bacteria were harvested by centrifugation at 17,700 × *g* for 10 min. For each 1 g (wet weight) of bacteria, 45 ml of 0.1 M Tris-hydrochloride buffer (pH 8.10) containing 200 mM NaCl and 0.02% (wt/vol) NaN_3 (Tris-saline) was used to suspend the cells. The outer membranes were extracted by the methods described by Johnston et al. (8), except that ethylenediaminetetraacetate was not included in any of the buffers. The extracts of each of the variants were centrifuged for 90 min at 100,000 × *g*, the pellets were resuspended in the starting buffer, and 4 M $MgCl_2$ was added to a final concentration of 10 mM. The membrane preparations were each treated with 0.1 mg of bovine pancreatic deoxyribonuclease I per ml, which had been dissolved (1 mg/ml) in water and treated with 0.1 mM phenylmethylsulfonyl fluoride before use. After digestion at 37°C for 30 min, the membrane preparations from the two variants were each applied to a column (180 by 2.5 cm) of Sepharose CL-6B (Sigma Chemical Co.) and eluted with Tris-saline. The material eluting at the void volume was collected and centrifuged at 100,000 × *g* for 90 min. The membranes were resuspended in Tris-saline buffer to a concentration of 1 mg of protein per ml.

Treatment of outer membranes with proteolytic enzymes. Assays to detect membrane-bound proteolytic fragments were performed as follows. A 1-ml sample (1 mg/ml) of each phenotypic variant was placed in each of four 10-ml tubes, yielding a total of eight samples (four containing membranes from O^- organisms and four containing membranes from O^+ organisms); 25 μg of each of the enzymes was added to a pair of tubes. One pair of tubes was used as controls. The preparations were incubated at 37°C for 30 min, diluted 1:10 with 0.25 M Tris-hydrochloride (pH 7.2), and centrifuged at 200,000 × *g* for 60 min. The pellet of each sample was suspended in 1 ml of Tris-saline buffer; 25 μl from each tube was removed and prepared for SDS-PAGE as described above.

To determine how proteolytic degradation varied with time, eight samples were prepared as described above. Starting at zero time, when the enzymes were added to the appropriate samples, and at 15, 30, 60, 90, 120, 240, and 360 min thereafter, 25-μl portions were removed from the eight samples and prepared for SDS-PAGE.

To examine the approximate location of the cleavage site of one enzyme compared with the cleavage site of another, two samples containing alpha-chymotrypsin and two samples containing TPCK-trypsin were incubated for 30 min. After 30 min, 20 μg of chymotrypsin was added to one of the samples containing TPCK-trypsin and 20 μg of TPCK-trypsin was added to one of the samples containing alpha-chymotrypsin; these preparations were incubated for an additional 30 min, and all samples were prepared for SDS-PAGE.

RESULTS

Effects of proteases on the growth of gonococci. Gonococcal strains and their opacity variants differed in growth inhibition when they

were exposed to proteolytic enzymes. Gonococci that had protein I species with 34K subunits exhibited growth inhibition by alpha-chymotrypsin greater than the inhibition observed with organisms that had protein I species of lower subunit molecular weights. O^- nonpiliated organisms with 32K protein I species were not inhibited by alpha-chymotrypsin, and gonococci with 33K protein I species were intermediate in growth inhibition (Table 1). In addition, the presence of protein II markedly enhanced the growth inhibition caused by alpha-chymotrypsin in nonpiliated O^+ strains with 34K or 33K protein I species. Nonpiliated organisms with 32K protein I species appeared to be resistant to alpha-chymotrypsin regardless of colonial opacity phenotype. Experiments in which pronase was used gave virtually identical results (data not shown). How these enzymes cause their inhibitory effects on gonococci is unknown, but the outer membranes of the organisms are most likely involved. The actions of trypsin, chymotrypsin, and pronase on the outer membrane proteins were examined both in intact organisms and in purified outer membrane vesicles from isogenic opacity variants of a number of strains.

Effects of proteases on gonococcal outer membrane proteins. (i) Protein I. Protein I is the single prominent protein species present on the surfaces of O^- gonococci. The subunit molecular weights of protein I species vary from strain to strain, as previously noted. Three strains were selected for such differences in protein I subunit size; these strains were used for comparing homogeneous nonpiliated O^- colonial forms and were incubated in alpha-chymotrypsin. Both unlabeled (Fig. 1A) and ^{125}I-labeled (Fig. 1B) gonococci were incubated with alpha-chymotrypsin, and the resulting preparations were then subjected to SDS-PAGE. For a comparison of this type to be valid, the concentrations of the substrate (protein I) had to be approximately equal in the three preparations. In both stained gels (Fig. 1A) and autoradiograms (Fig. 1B) from gels of ^{125}I-labeled gonococci, several observations were made. First, equivalent numbers of bacteria of the three strains contained similar amounts of protein I,

TABLE 1. *Relationship between colony opacity and growth inhibition by chymotrypsin for nonpilated gonococci*

Strain	Colony opacity	Protein I subunits[a]	Inhibition zone diam (mm)[b]
F62	O^+	34K	7
F62	O^-	34K	4
10677-2	O^+	33K	4
10677-2	O^-	33K	2
120176-2	O^+	32K	0
120176-2	O^-	32K	0

[a] As determined by SDS-PAGE.

[b] Zone size was the total diameter minus the disk diameter (6 mm).

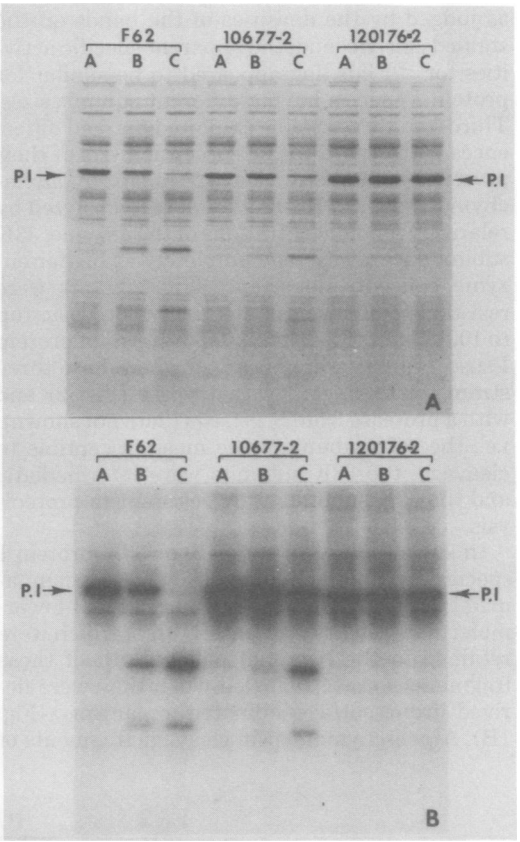

FIG. 1. *Nonpiliated O^- variants from three strains were selected because of the different subunit sizes of their protein I (P.I) species: strain F62, 34K; strain 10677-2, 33K; and strain 120176-2, 32K. These organisms were radioiodinated with Iodogen, as described in the text. Intact organisms were incubated with buffer alone (lanes A) or with two concentrations of alpha-chymotrypsin (lanes B, 2 μg/ml; lanes C, 20 μg/ml) and were then pelleted by centrifugation and prepared for SDS-PAGE. Both the Coomassie blue-stained gel (A) and the autoradiogram (B) show the differing subunit forms of protein I, as observed in the buffer controls. In comparing lanes B of the three organisms, note that the protein I expressed in strain F62 (34K) is greatly reduced compared with the protein I of strain 10677-2 (33K) and the protein I of strain 120176-2 (32K). Also, compare lanes C of strains 10677-2 (33K) and 120176-2 (32K) and note that when the concentration of alpha-chymotrypsin was increased, protein I of strain 10677-2 (33K) was affected and protein I of strain 120176-2 (32K) remained resistant and equal in intensity to protein I of the buffer control. The two fragments resulting from cleavage of the 34K and 33K protein I species are evident in lanes B and C of strains F62 and 10677-2.*

as judged by the densities of the bands on the stained gels. Second, the apparent specific activities for ^{125}I labeling appeared to be similar for protein I species having different subunit sizes. Third, the three strains exhibited marked differences with regard to the susceptibilities of their protein I subunits to cleavage in situ by alpha-chymotrypsin; 34K subunits were hydrolyzed by relatively low concentrations of the enzyme, 33K subunits were cleaved completely at higher enzyme concentrations, and 32K subunits were resistant to all levels of alpha-chymotrypsin (up to 100 μg/ml) (Fig. 1). Similar patterns of protein I susceptibility were observed when these three strains were treated with trypsin (Fig. 2) and with a protease from *S. griseus* (data not shown); i.e., the 34K subunits were most susceptible to cleavage, the 33K subunits were intermediate, and the 32K subunits were resistant to proteolysis.

In situ cleavage of the 34K or 33K protein I species by alpha-chymotrypsin was accompanied by the appearance of two prominent lower-molecular-weight fragments, both of which were retained in the bacterial pellet. Both of these fragments were ^{125}I labeled when they were derived from surface-iodinated organisms (Fig. 1B). Alpha-chymotrypsin cleavage fragments of the 34K or 33K protein I subunits were identical in size when O^+ and O^- colony forms of the same strain were compared (Fig. 3). However, hydrolysis of protein I subunits by alpha-chymotrypsin was more extensive at a given protease concentration for O^+ colony forms than for O^- colony forms from the same strain; this differential susceptibility of O^+ and O^- preparations was observed for gonococci containing 34K or 33K protein I subunits and was also found when organisms were treated with trypsin (Fig. 2) or *S. griseus* protease (data not shown). When isolated outer membranes were incubated with alpha-chymotrypsin, the relative susceptibilities of the 34K, 33K, and 32K protein I subunits to enzymatic cleavage were the same as the relative susceptibilities observed with intact gonococci. Both 34K and 33K protein I subunits were cleaved into two prominent fragments, and both of these fragments were retained in the outer membranes (Fig. 4). When the two fragments were subjected to ^{125}I-labeled peptide mapping, the iodinated peptides obtained accounted for all of the peptides observed when native protein I was mapped. As with 32K protein I in intact gonococci, 32K protein I in purified outer membranes was resistant to proteolysis (Fig. 4). Alpha-chymotrypsin cleavage of 34K protein I pro-

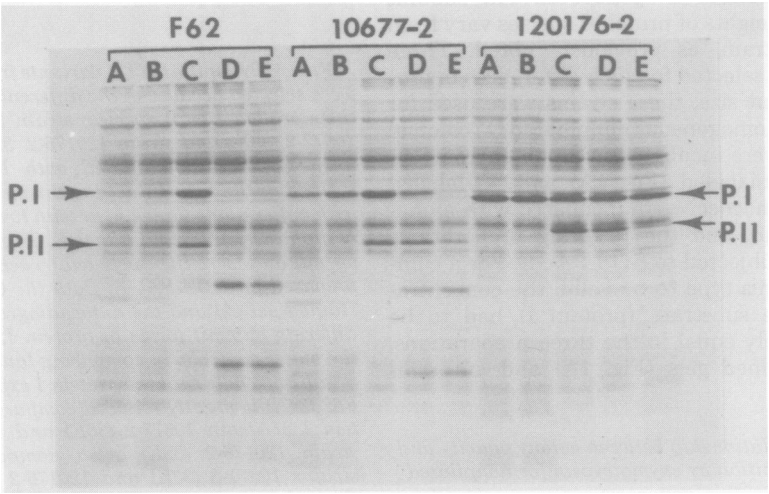

FIG. 2. *Nonpiliated O^+ variants of strains F62, 10677-2, and 120176-2 were incubated with buffer (lanes C), with trypsin (lanes A, 500 μg/ml; lanes B, 250 μg/ml), or with alpha-chymotrypsin (lanes D, 10 μg/ml; lanes E, 100 μg/ml) before analysis by SDS-PAGE. In addition to their protein I (P.I) species, these O^+ organisms contained a prominent protein (protein II [P.II]) whose presence was correlated with colony opacity. The protein II species of these three strains were hydrolyzed readily by trypsin, regardless of the relative susceptibility of the protein I on the same organism to cleavage by the same enzyme. However, the cleavage of protein II by alpha-chymotrypsin was most apparent in strain F62 (34K), whose protein I was cleaved readily by this protease. Protein II of strain 120176-2, whose 32K protein I was resistant to alpha-chymotrypsin, was relatively resistant to the action of the same enzyme. Both the protein I (33K) and the protein II of strain 10677-2 were intermediate in relative susceptibility to alpha-chymotrypsin hydrolysis compared with the proteins of the other two strains.*

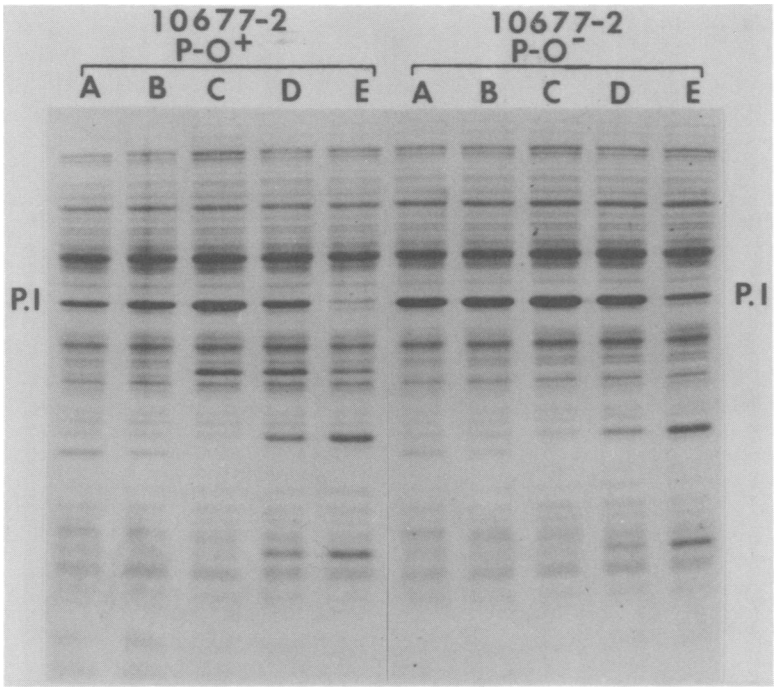

FIG. 3. *Nonpiliated O^+ and O^- variants of strain 10677-2 (33K protein I [P.I]) were incubated with buffer (lanes C), with trypsin (lanes A, 500 μg/ml; lanes B, 250 μg/ml), or with alpha-chymotrypsin (lanes D, 10 μg/ml; lanes E, 100 μg/ml) before SDS-PAGE. Note the apparently greater susceptibility of protein I of nonpiliated O^+ gonococci to alpha-chymotrypsin cleavage compared with protein I of nonpiliated O^- cells from the same strain. However, the apparent molecular weights of the resulting fragments from protein I were the same for both phenotypes.*

duced 22K and 14K fragments (Fig. 5). After prolonged enzymatic digestion with alpha-chymotrypsin (3 to 4 h), there was a secondary split of the 22K fragment, and a slightly smaller fragment appeared. TPCK-trypsin appeared to sever this protein at two separate sites, producing three pieces; one of these was a 10K fragment, one was a 21K fragment, and one was a small soluble fragment which did not remain with the membrane (Fig. 5). We could differentiate these two sites because TPCK-trypsin showed preference for one site over the other, initially producing 28K and 10K fragments. The 28K fragment contained a secondary site of trypsin action, and as the reaction went to completion, the trypsin further cleaved this larger fragment. One 21K fragment remained associated with the membranes, and the other smaller fragment was soluble and remained in the supernatant. The 28K fragment also appeared to contain the alpha-chymotrypsin cleavage site because, when alpha-chymotrypsin was added after trypsin cleavage, this larger segment was reduced to the 22K fragment (Fig. 6). The pronase digests were similar to the TPCK-trypsin digests but showed no preference to produce the large 28K fragment (Fig. 5).

When the control samples which did not have any extrinsically added enzymes were examined carefully, there appeared to be protein bands in the SDS-PAGE pattern which were very similar to the fragments produced by alpha-chymotrypsin digestion (Fig. 5). If these samples were incubated for several hours, there was an obvious diminution of the major protein band and commensurate increases in the degradation products. In fact, in strain MS11/020780 this endogenous enzyme appeared to be particularly active, such that the purified outer membrane preparations contained essentially no protein I but did contain large amounts of the two degradation products. ^{125}I-labeled peptide mapping revealed that these bands from buffer-incubated outer membranes were identical to the bands from alpha-chymotrypsin-treated specimens. The enzyme did not appear to be inhibited by phenylmethylsulfonyl fluoride, a noncompetitive inhibitor of serine esterases.

(ii) Protein II. Protein II species were usually hydrolyzed at lower alpha-chymotrypsin, trypsin, and *S. griseus* protease concentrations than were required to cleave protein I in the same

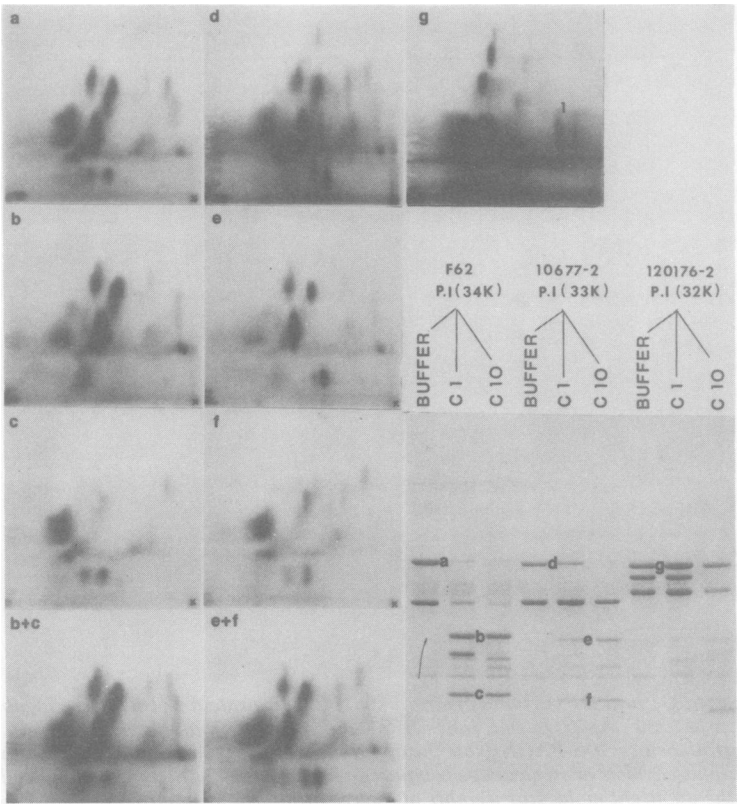

FIG. 4. *^{125}Iodine peptide mapping of protein I (P.I) and the fragments produced by in situ cleavage with alpha-chymotrypsin. Isolated outer membranes from three representative strains (strains F62, 10677-2, and 120176-2) were incubated with buffer, with 1 μg of alpha-chymotrypsin (C1) per ml, or with 10 μg of alpha-chymotrypsin (C10) per ml, pelleted, and subjected to SDS-PAGE. After staining, selected protein or peptide bands were excised for ^{125}I-labeled peptide mapping. (a, d, and g) Peptide maps of intact protein I species. (b and c) Fragments resulting from chymotrypsin cleavage of 34K protein I. (e and f) Fragments from 33K protein I. Compared with the maps in (a) and (d), superimposition of the maps in (b) and (c) and superimposition of the maps in (e) and (f), respectively, show that together these maps appear to contain all of the radioiodinated peptides present in the intact proteins I subunits.*

organisms. Protein II subunits also varied in their relative susceptibilities to cleavage, depending on the protein I subgroup of the strain. Protein II appeared to be cleaved more readily in situ in gonococci with 34K protein I than in gonococci with 33K or 32K protein I (Fig. 2). After enzymatic treatment, only one fragment remained bound to the membrane. Treatment with trypsin or pronase produced a membrane-bound peptide of approximately 6K, whereas the peptide resulting from alpha-chymotrypsin treatment was a 19K peptide (Fig. 5).

We found that membranes from O^+ variants incubated without protease contained a fragment having a molecular weight identical to the molecular weight of a fragment resulting from alpha-chymotrypsin digestion. This fragment was not observed in enzyme-treated or untreated membranes derived from O^- variants.

(iii) Protein III. Protein III was present in the membranes of all variants and strains and can be defined as the protein with apparent molecular weights of 30,000 in the absence of mercaptoethanol and 31,000 in the presence of mercaptoethanol; it is not heat modifiable (15). This protein was completely resistant to all of the enzymes used in this study.

Lack of bactericidal activity of alpha-chymotrypsin. Our results stimulated attempts to kill gonococci directly with proteolytic enzymes in vitro. When an organism expressing either 34K protein I (strain F62) or 33K protein I (strain 10677-2) was exposed to alpha-chymotrypsin in a concentration adequate to cleave the major protein of the organism completely, no discernible reduction in colony-forming units

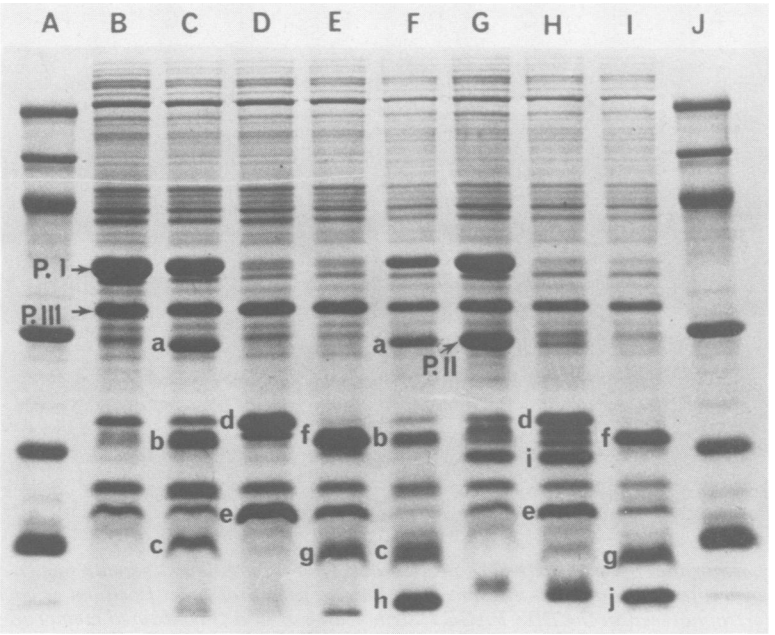

FIG. 5. *SDS-PAGE analysis of purified outer membranes from O⁻ (lanes B through E) and O⁺ (lanes F through I) variants of strain R10 (34K protein I) to which 25 μg of trypsin (lanes C and F) per ml, 25 μg of alpha-chymotrypsin (lanes D and H) per ml, and 25 μg of pronase (lanes E and I) per ml was added. These preparations were incubated at 37°C for 30 min. The membranes were then diluted 1:10 and centrifuged at 200,000 × g for 60 min. The pellets were suspended, and a portion of each was subjected to SDS-PAGE. Lanes B and G contained membranes to which only buffer was added. The markers in lanes A and J were as follows, from top to bottom: human transferrin (80K), catalase (60K), ovalbumin (45K), carbonic anhydrase (30K), soybean trypsin inhibitor (21.5K), and cytochrome c (12.5K). The protein I (P.I) species of the O⁺ and O⁻ variants migrated identically and the O⁺ control contained protein II (P.II). All samples contained protein III (P.III). The peptide fragments of protein I are identified on the figure by lower-case letters as follows: a, b, and c are trypsin fragments of protein I; d and e are chymotrypsin fragments of protein I; f and g are pronase fragments of protein I; and h, i, and j are trypsin, chymotrypsin, and pronase fragments of protein II, respectively. In the control samples of both O⁻ and O⁺ variants there were peptides which migrated in the same positions as the chymotrypsin fragments of protein I and, in the case of the O⁺ variant, of protein II. Lanes C and F also show that in the presence of protein II, the degradation of protein I by trypsin was increased greatly.*

was observed compared with organisms incubated in buffer alone. The addition of alpha-chymotrypsin at an early log phase of growth of gonococci in defined liquid medium (20) and the subsequent growth of the gonococci, as followed by optical density, revealed that regardless of the species of protein I, the gonococci continued to multiply and reached the same optical density as gonococci grown in the absence of the enzyme. An SDS-PAGE analysis of the organisms grown in the presence of alpha-chymotrypsin revealed that those organisms expressing 34K or 33K protein I lacked any discernible intact protein I.

DISCUSSION

We used proteolytic enzymes to probe the surface structures of gonococcal outer membrane proteins, and our study revealed the following. First, at least portions of two species of major outer membrane protein (protein I) and the opaque protein (protein II) are exposed on the membrane surface and interact with proteolytic enzymes, confirming the observations of several workers who used other techniques (6, 8, 9, 15, 18). Because protein I species having 34K and 33K subunits are susceptible to cleavage in situ by proteolytic enzymes, the use of proteolytic enzymes in the purification of gonococcal outer membranes and their proteins should be avoided, and in fact, it may be advantageous to add protease inhibitors in the course of purification. Second, protein I seems to be tightly associated with the unit membrane in at least two segments. In each case where a 34K or 33K protein I species was cleaved by an enzyme, two of the resulting fragments remained closely associated with the membrane after ultracentrif-

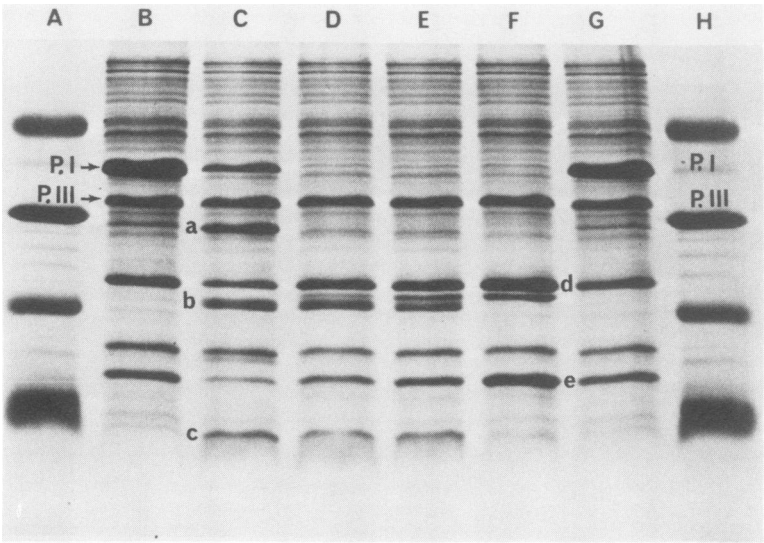

FIG. 6. *Outer membranes of strain R10 (34K protein I [P.I]) were preincubated with one enzyme for 30 min at 37°C and then treated with another enzyme for the same period of time. Portions of each sample were prepared and electrophoresed in the SDS-PAGE system. Lanes B and G contained membranes incubated in buffer. Lane C contained membranes treated with TPCK-trypsin (20 μg/ml) for 60 min. In lane D, the membranes were subjected first to TPCK-trypsin (20 μg/ml) for 30 min; then, alpha-chymotrypsin (20 μg/ml) was added, and the incubation was carried out for an additional 30 min. The results of the reverse experiment (i.e., first alpha-chymotrypsin treatment and then trypsin treatment) are shown in lane E. Lane F contained membranes incubated for 60 min with alpha-chymotrypsin (20 μg/ml) alone. The markers (lanes A and G) were as follows: ovalbumin (45K), carbonic anhydrase (30K), soybean trypsin inhibitor (21.5K), and cytochrome c (12.5K). The 28K fragment resulting from the cleavage of protein I is present in lane C. After treatment with alpha-chymotrypsin, this band was lost, and there was an increase in the 22K band. The fragments produced by the apparent endogenous enzyme were present in the buffer controls (lanes B and G). P.III, Protein III.*

ugation. It appears that protein I resembles a hairpin with both ends inserted in the membrane and that these ends are joined by a surface-exposed region, as shown in Fig. 7. It is within this looped portion that the sites susceptible to proteolysis are located. Our study does not explain why the 32K protein I subunits are resistant to in situ proteolysis. Two plausible explanations are (i) the surface-exposed region of 32K protein I lacks peptide sequences susceptible to proteolysis, and (ii) sensitive peptide sequences exist, but are hindered sterically from reacting with proteolytic enzymes. The latter hypothesis may seem to be difficult to reconcile with the ^{125}I labeling data, in which both the 34K and 32K subunits appear to be iodinated with equal efficiency. However, our studies indicate that protein I has porin-like properties (Greco et al., Fed. Proc. **39**:1813, 1980), suggesting that protein I forms a hydrophilic channel of definite size through the membrane. This would allow small ionic species, such as the "activated iodine" generated by the labeling procedure, to penetrate into the channel and to react with tyrosyl residues located there. On the other hand, the high-molecular-weight proteases would be excluded from the channel. Whatever the explanation for the differences in susceptibility to in situ enzymatic digestion, our data suggest that the surface-exposed portions of the 34K and 33K protein I species are substantially different than those of the 32K protein I species. Third, protein II is cleaved more readily by exogenous proteases than protein I. In contrast to protein I, protein II appears to be associated with the membrane in only one segment (Fig. 7); the remainder is solubilized upon enzyme digestion. Fourth, the presence of protein II has a synergistic effect on the proteolysis of protein I (34K or 33K species); e.g., the 34K protein I was cleaved more readily in the presence of protein II (34K O^+) than in the absence of protein II (34K O^-). Also, the reverse seemed to be true; protein II appeared to be degraded more readily in the presence of proteolytic-susceptible protein I (34K O^+) than in the presence of nonsusceptible protein I (32K O^+). A priori, it seems by virtue of there being an increased number of proteins susceptible to proteolytic attack and a constant number of enzyme molecules available

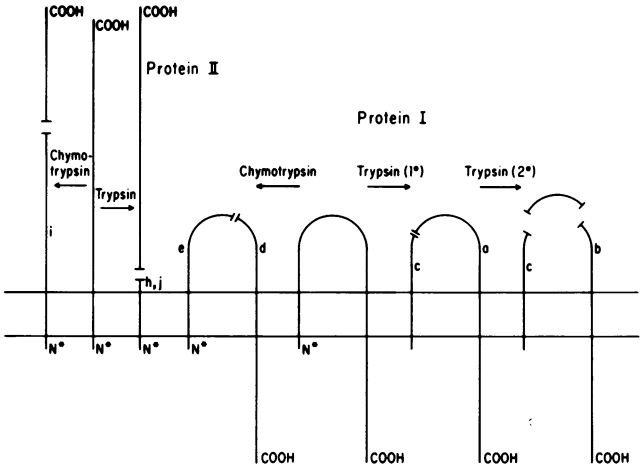

FIG. 7. *Model showing the hypothetical structure of protein I and protein II within gonococcal outer membranes and the action of trypsin and alpha-chymotrypsin on the two proteins. The fragments (a through i) were derived from experiments shown in Fig. 5 and 6. Protein I appears to have a looplike structure, with two segments of the molecule embedded within the membrane. Chymotrypsin splits protein I in the exposed region, leaving two portions (fragments d and e) of the molecule within the membrane. Trypsin cleaves protein I at two sites (one preferentially), initially giving fragments a and c. As the reaction continues, fragment a is cleaved once more, releasing a small soluble piece, and this leaves segments b and c within the membrane. Protein II seems to be associated with the membrane in only one portion of its structure. One fragment of protein II remains with the membrane after either chymotrypsin treatment (fragment i) or trypsin digestion (fragment h).*

for cleavage that the exact opposite would be observed. However, it has been shown that both trypsin and alpha-chymotrypsin undergo substrate activation in the presence of two substrates, such as casein and gelatin, with an increased velocity of reaction which does not conform to Michaelis kinetics (2, 3). Fifth, there seems to be a strong correlation between the degree of protease-induced growth inhibition observed on a solid medium with a particular strain and the susceptibility of its protein I. This correlation suggests that hydrolytic disruption of the protein I subunit, either directly or indirectly, leads to a bactericidal effect on the organism. However, the absence of clear-cut killing of gonococci by alpha-chymotrypsin despite total cleavage of the bacterial protein I subunits suggests that the action of the enzyme is not a direct one. This is also suggested by the finding that protein I and protein II fragments were present in buffer controls to which no exogenous protease was added. This suggests the presence of an endogenous enzyme which is capable of cleaving outer membrane proteins. The existence of such an enzyme has been noted by others (1). Preliminary experiments with purified 34K protein I in planar lipid bilayers have indicated that the enzymatic cleavage of this protein causes a dramatic change in its porin-like properties, as measured by this technique (F. Greco et al., unpublished data). However, alpha-chymotrypsin digestion of protein I subunits followed by exposure of the intact gonococci to either lysozyme or crystal violet has not indicated an increased porosity of the protease-treated organisms (P. Barrera and J. Swanson, unpublished data).

ACKNOWLEDGMENTS

This work was supported in part by Public Health Service grant AI 10615 from the National Institutes of Health and by a grant from the Rockefeller Foundation for the Support of Reproductive Biology. M.S.B. was supported by Public Health Service fellowship AI 05594 from the National Institutes of Health.

LITERATURE CITED

1. **Cannon, J. G., D. G. Klapper, E. Y. Blackman, and P. F. Sparling.** 1980. Genetic locus (*nmp*-1) affecting the principal outer membrane protein of *Neisseria gonorrhoeae*. J. Bacteriol. **143**:847-851.
2. **Castañeda-Agullõ, M., and L. M. Del Castillo.** 1958. The action of alpha-chymotrypsin upon mixtures of esters and proteins at enzyme-saturating concentrations. J. Gen. Physiol. **42**:49-68.
3. **Castañeda-Agullõ, M., and L. M. Del Castillo.** 1958. Simultaneous hydrolysis of esters and proteins at saturation levels. J. Gen. Physiol. **41**:485-500.
4. **Draper, D. L., J. F. James, G. F. Brooks, Jr., and R. L. Sweet.** 1980. Comparison of virulence markers of peritoneal and fallopian tube isolates with endocervical *Neisseria gonorrhoeae* isolates from women with acute salpingitis. Infect. Immun. **27**:882-888.
5. **Elder, J. H., R. A. Pickett, J. Hampton, and R. A.**

Lerner. 1977. Radioiodination of proteins in single polyacrylamide gel slices. J. Biol. Chem. **252:**6510–6515.

6. **Heckels, J. E.** 1977. The surface properties of *Neisseria gonorrhoeae*: isolation of the major components of the outer membrane. J. Gen. Microbiol. **99:**333–341.
7. **James, J. F., and J. Swanson.** 1978. Studies on gonococcus infection. XIII. Occurrence of color/opacity colonial variants in clinical cultures. Infect. Immun. **19:** 332–340.
8. **Johnston, K. H., and E. C. Gotschlich.** 1974. Isolation and characterization of the outer membrane of *Neisseria gonorrhoeae*. J. Bacteriol. **119:**250–257.
9. **Johnston, K. H., K. K. Holmes, and E. C. Gotschlich.** 1976. The serological classification of *Neisseria gonorrhoeae*. I. Isolation of the outer membrane complex responsible for serotypic specificity. J. Exp. Med. **143:** 741–758.
10. **Kellogg, D. S., Jr., I. R. Cohen, L. C. Norins, A. L. Schroeter, and G. Reising.** 1968. *Neisseria gonorrhoeae*. II. Colonial variation and pathogenicity during 35 months in vitro. J. Bacteriol. **96:**596–605.
11. **Kellogg, D. S., Jr., W. L. Peacock, Jr., W. E. Deacon, L. Brown, and C. I. Pirkle.** 1963. *Neisseria gonorrhoeae*. I. Virulence genetically linked to colonial variation. J. Bacteriol. **85:**1274–1279.
12. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.
13. **Lambden, P. R., J. E. Heckels, L. T. James, and P. J. Watt.** 1979. Variations in surface protein composition associated with virulence properties in opacity types of *Neisseria gonorrhoeae*. J. Gen. Microbiol. **114:**305–312.
14. **Markwell, M. A. K., and C. F. Fox.** 1978. Surface-specific iodination of membrane proteins of viruses and eucaryotic cells using 1,3,4,6-tetrachloro-α,6α-diphenylglycoluril. Biochemistry **17:**4807–4817.
15. **McDade, R. L., Jr., and K. H. Johnston.** 1980. Characterization of serologically dominant outer membrane proteins of *Neisseria gonorrhoeae*. J. Bacteriol. **141:** 1183–1191.
16. **Salit, I. E., and E. C. Gotschlich.** 1978. Gonococcal color and opacity variants: virulence for chicken embryos. Infect. Immun. **22:**359–364.
17. **Spector, T.** 1978. Refinement of the Coomassie blue method of protein quantitation. Anal. Biochem. **86:**142–146.
18. **Swanson, J.** 1978. Studies on gonococcus infection. XII. Colony color and opacity variants of gonococci. Infect. Immun. **19:**320–331.
19. **Swanson, J.** 1979. Studies on gonococcus infection. XVIII. ^{125}I-labeled peptide mapping of the major protein of the gonococcal cell wall outer membrane. Infect. Immun. **23:**799–810.
20. **Wong, T. P., R. K. Shockley, and K. H. Johnston.** 1980. WSJM, a simple chemically defined medium for the growth of *Neisseria gonorrhoeae*. J. Clin. Microbiol. **11:**363–369.