INFECTION AND IMMUNITY, July 1978, p. 292-302
0019-9567/78/0021-0292$02.00/0

Vol. 21, No. 1
Printed in U.S.A.

Studies on Gonococcus Infection

XIV. Cell Wall Protein Differences Among Color/Opacity Colony Variants of *Neisseria gonorrhoeae*

JOHN SWANSON

Departments of Pathology and Microbiology, University of Utah College of Medicine, Salt Lake City, Utah 84132

Received for publication 20 January 1978

Gonococci from colonies exhibiting optical opacity and dark coloration have surface proteins that are not visualized in isogenic transparent, light-colored colony forms. These "colony opacity-associated proteins" have apparent molecular weights varying from 24,000 to 30,000 by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate; their apparent molecular weights are independent of that for their major outer membrane protein. The opacity-associated proteins are more susceptible to hydrolysis by trypsin than is the major outer membrane protein, but gonococci possessing the opacity-associated protein(s) also show enhanced susceptibility of their major outer membrane proteins to the action of trypsin. These conclusions were reached by comparing the electrophoretic patterns of whole-cell lysates from both "laboratory strains" and several recent clinical isolates of *Neisseria gonorrhoeae*.

Neisseria gonorrhoeae grown on solid, transparent medium can exhibit a broad variety of colonial forms. Raven (15), as well as Morton and Shoemaker (13), noted small-colony variants which were later categorized as type 1 or 2 colonies by Kellogg et al. (10). These small type 1 and 2 colonies were shown to be more virulent for human volunteers (9), to predominate in clinical isolates from males and females (17), and to contain pilus-bearing gonococci (6, 20). The larger type 3 and 4 colonies are generated by nonselective serial passage in vitro, are much less virulent in male volunteers, and contain nonpilated organisms.

The coloration of gonococcal colonies has also been noted to vary widely in cultures on solid medium (1, 2, 5, 10). Colony color was included as one criterion for colony classification in the Kellogg scheme, where type 2 and 3 colonies were somewhat darker than types 1 and 3. An additional form, type 5, consists of very dark, large colonies (1, 5). Gonococcal colony color was studied in somewhat greater detail in a previous report (19). In that study, colony darkness was correlated with a high degree of intergonococcal aggregation, and these colonies also displayed optical opacity. The action of trypsin in disaggregating and killing gonococci from dark, opaque, highly aggregated colony preparations suggested that these organisms possessed cell surface proteins that differed qualitatively or quantitatively from isogenic gonococci in light-colored, transparent colonies. Preliminary studies on gonococcal strains passaged for several years in vitro suggested the presence of additional proteins on the dark-colored, opaque colonies. In the present study, colony color/opacity variants from both recent clinical isolates and laboratory strains are compared by slab gel electrophoresis of whole-cell lysates. Lactoperoxidase-catalyzed ^{125}iodination of proteins on intact gonococci was utilized to aid identification of gonococcal cell wall surface proteins and to study the susceptibilities of these proteins to trypsin.

MATERIALS AND METHODS

Gonococci. Laboratory strains F62 and MS11, as well as fresh clinical isolates of gonococci, were used and were grown at 36°C in a 5% CO_2 atmosphere on a modified, clear typing agar described previously (19). All clinical specimens were initially isolated on Thayer-Martin medium (21) and were nonselectively transferred to typing agar. After this first passage, colonies with the desired color/opacity characteristics were selected if present. After two to four additional selective, single-colony passages, the gonococci were utilized for study by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Colony lighting and microscopy. Gonococcal colonies were examined by stereomicroscopy as described in detail previously (19). Briefly, with a stereomicroscope the diffusing, substage reflector is utilized to determine colony color and edge morphology. If the polished mirror is used with the stereomicro-

scope, opacity of the colony can be judged. The method previously described for examination of colonies by reflected lighting was also utilized (14).

SDS-PAGE. Colonies from 20- to 23-h cultures were removed with a swab and suspended in phosphate-buffered saline, pH 7.4 (PBS), to an optical density of 0.4 at 500 nm or to a turbidity of 100 Klett units (blue filter, Klett-Summerson colorimeter). A 1.5-ml amount of this suspension was centrifuged (Microfuge B, Beckman Instruments, Inc., Fullerton, Calif.) at top speed for 5 min, and the pellet was suspended in 100 μl of SDS solubilizing solution (11). After the SDS-gonococci mixture was boiled for 10 min, the lysate was subjected to SDS-PAGE with the buffer system described by Laemmli (11). No SDS was incorporated into either the 12.5% separating gel or the 4% stacking gel (M. Wycoff, R. Rodbard, and A. Chrambach, Fed. Proc. **35**:1383, 1976). Electrophoresis in tris(hydroxymethyl)aminomethane (Tris)-glycine (pH 8.6) buffer containing 0.1% SDS (BDH Chemicals Ltd.) was carried out at a constant current of 40 mA. On completion of electrophoresis, the gel was removed, fixed for several hours in 7% acetic acid–25% isopropanol, stained with 0.02% Coomassie brilliant blue R in acetic acid-isopropanol for 1 h, and destained by diffusion in acetic acid-isopropanol. Autoradiograms were obtained either with a wet gel wrapped in Saran Wrap or with a dried gel placed in contact with X-ray film (XM-2, Kodak).

^{125}Iodination of whole gonococci. Intact gonococci were labeled by a modification of the method of Marchalonis et al. (12). A 1.5-ml amount of this suspension of gonococci in PBS with an optical opacity of 250 Klett units (Klett-Summerson colorimeter, blue filter) was centrifuged (Microfuge B, Beckman Instruments, Inc.) at top speed for 5 min, and the pellet was suspended in 25 μl of ^{125}I (New England Nuclear Corp., Boston, Mass.; high specificity activity, carrier-free; approximately 1 mCi/ml in 10^{-5} M KI) and 25 μl of lactoperoxidase (Sigma Chemical Co., St. Louis, Mo.). To this suspension was added 10 μl of 0.3% H_2O_2 (Mallinkrodt Chemical Co., St. Louis, Mo.; obtained as a fresh 30% solution approximately each month and refrigerated) every 3 min during the 12-min incubation at room temperature. A 1-ml amount of cold PBS containing 5 mM cysteine was added, and the gonococci were washed once more in PBS-cysteine. This pellet was suspended in 50 μl of SDS-solubilizing solution if only SDS-PAGE and autoradiography were needed. When used in trypsinization studies, the pellet was suspended in 0.046 M Tris–0.0115 M $CaCl_2$, pH 9.1. The exact volume used for this dilution varied slightly depending on how many ^{125}I-labeled portions were to be utilized in succeeding steps, but, for example, it was usually 300 μl if two portions were needed from each radiolabeled preparation or 600 μl in cases in which five portions were desired. In the latter cases, the amounts of gonococci used for iodination as described above had been doubled.

Incubation of ^{125}I-labeled gonococci with trypsin. Portions (100 μl) of the ^{125}I-labeled gonococci in Tris-$CaCl_2$ buffer noted above were dispensed into 400-μl Microfuge tubes (Beckman Instruments, Inc.), and to each was added 100 μl of buffer. A 50-μl amount of trypsin (trypsin-tolylsulfonyl phenylalanyl chloromethyl ketone [TPCK]; Worthington Biochemicals Corp., Freehold, N.J.; 0.5, 1.5, or 10 μg/ml) in the same buffer was added to the other tubes to yield enzyme concentrations of 0.1, 0.2, 1, or 2 μg/ml. Higher amounts of trypsin were sometimes utilized, as noted in the text. The Microfuge tubes were then placed in a Biogamma gamma spectrometer (Beckman Instruments, Inc.) and counted for ^{125}I. The counting period was also an incubation period with trypsin and was continued for 25 min at which time 50 μl of a solution of trypsin soybean inhibitor (Sigma Chemical Co., St. Louis, Mo.; 1 mg/ml) was added and the specimens were centrifuged (Microfuge, full speed, 5 min). The supernatants were carefully sampled (two samples, 50 μl each), the remainder of the supernatant was aspirated, and 25 μl of SDS-PAGE solubilization solution was added to each pellet. The supernatant portions and the contents of the pellet-containing Microfuge tube were then counted in a gamma counter.

RESULTS

Gonococcal colony morphology. The variety of morphological characteristics found in gonococcal colonies on solid medium have been described in several previous studies. Colonial size, edge definition, granularity, opacity, and consistency formed the basis for Kellogg's classification into four types—T1, T2, T3, and T4—each of which is characterized by particular combinations of the morphological features noted (10). I have suggested that colonies of pilated gonococci be designated as P+ (T1-like) or P++ (T2-like) on the basis of whether the colony has a rather indistinct or a very definite edge, respectively. All colonies of nonpilated organisms (T3- or T4-like) are called P−.

When P+, P++, or P− colonies were examined by stereomicroscopy after 22 to 24 h of incubation, a variety of colony color and opacity forms were observed. Colony color ranged from very dark brown-black to almost completely colorless. Colony opacity varied from completely opaque, yellowish colonies to almost totally transparent, slightly blue forms if a polished mirror was used for illumination. The correlations between colony color and colony opacity are noted in an earlier report (18). Designations used for colony color are extra dark, dark, light, and extra light. Colony opacity will be indicated as follows: opaque, opaque/transparent, transparent/opaque, and transparent. Typical P++ colonies with dark, opaque and light, transparent characteristics are shown in Fig. 1. These same colonies are also seen when illuminated from above at an angle of approximately 40 to 45°, as described by Penn et al. (14). P++ dark, opaque colonies showed double highlights as do P+ dark, opaque ones; this pattern was consistently obtained with a variety of light incidence angles (approximately 30 to 60°). Depending on the angle used for lighting, P++ light, transpar-

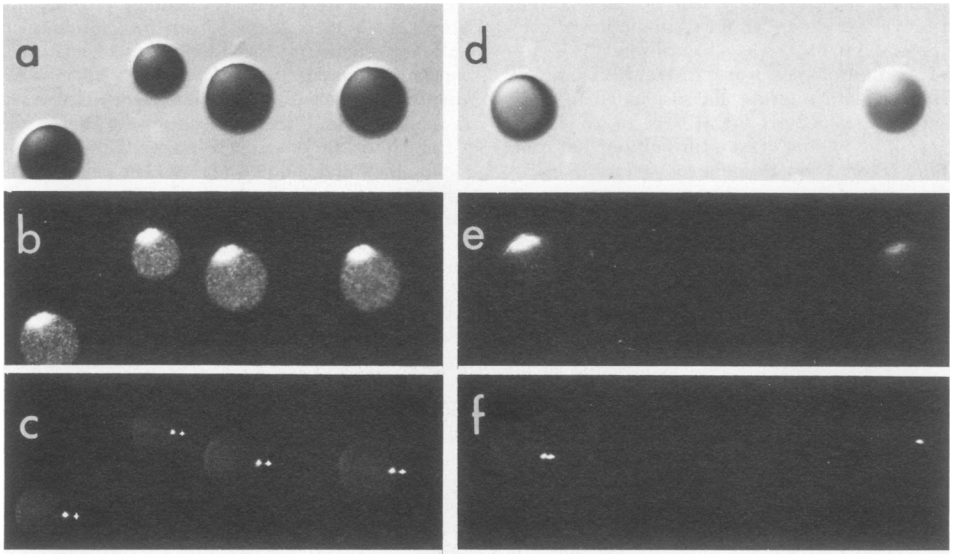


FIG. 1. *Appearances of gonococcal colonies are shown with three different illumination techniques: (a) and (d) utilize transmitted illumination from a substage diffusing surface; (b) and (e) use light transmitted from a substage polished mirror; (c) and (f) show reflecting foci from colonies illuminated from above the agar surface (approximately 45° to place of agar). (a), (b), and (c) are of the same P++ opaque colonies. These have distinct edges and are very dark in (a), appear opaque in (b), and exhibit double highlight reflecting patterns in (c). In (d), (e), and (f), P++ (left) and P+ (right) colonies are shown. Note the more distinct edge of the P++ colony in (d) as compared with the P+ form. Both are transparent in (e). The P++ transparent colony shows double highlight reflection, whereas the P+ transparent form exhibits a single reflecting focus in (f).*

ent colonies exhibited double highlights (angle greater than approximately 45°) or single highlights (less than 40°), whereas P+ light, transparent colonies showed either single (>45°) or no highlights (<40°). P− colonies had no highlight reflections except when very high angles of lighting were used (>55°).

The colony characteristics noted above pertain to cultures examined after approximately 1 day of incubation. The differences in colony opacity seen after 1 day were not present if the cultures were incubated at either room temperature or at 36°C for an additional 24 h, at which point all colonies assumed a rather opaque appearance. After the additional day of incubation, those colonies that had been opaque after 1 day retained their integrity and could be picked up with a loop or pushed about the agar surface. Colonies that had transparent characteristics at 22 to 24 h could not be removed from the medium surface as they had a tenacious stringy consistency and adhered to the agar after the additional incubation.

Comments on SDS-PAGE of gonococcal lysates. Protein banding patterns shown in the accompanying figures were all obtained with whole gonococci lysed by boiling in SDS. Use of whole-cell lysates presents potential limitations in observing small banding differences among many different proteins and in discerning differences in protein bands that may be superimposed on others. Despite these limitations, whole-cell lysates were utilized for this study because of the possibility that some proteins might be solubilized and lost during more involved preparation procedures.

Some difficulty was encountered in obtaining consistent SDS-PAGE patterns for protein species smaller than about 20,000 daltons (20K). Part of this problem was resolved by "fixing" the slab gel for several hours before Coomassie brilliant blue staining. This procedure appeared to produce somewhat more intense staining of all bands and gave the most uniform results found so far for the smaller gonococcal proteins. Sharp bands that were reasonably well separated in the molecular weight range under current study were obtained with the SDS-Tris-glycine system described by Laemmli (11) in 12.5% acrylamide gels.

The number of protein bands visualized by staining or autoradiography was somewhat variable depending on the amount of SDS lysate loaded onto the gel and on the extent of iodination. Additional difficulty was encountered in some autoradiograms because one heavily emit-

ting species obscured the presence of others with similar molecular weights.

SDS-PAGE patterns for gonococci in general. SDS-PAGE examination of numerous colony preparations from several different strains of gonococci revealed the following results. (i) All gonococci had identical patterns by SDS-PAGE for proteins larger than their major outer membrane protein (MOMP). (ii) The apparent molecular weight of gonococcal MOMP varied from strain to strain as previously noted by Johnston et al. (8). (iii) A total of 10 to 20 emitting bands were seen by autoradiography of gonococci ^{125}I labeled as whole cells. This variability in number was influenced by the variables mentioned above.

SDS-PAGE of opaque and transparent variants in recent isolates. A total of 22 recent gonococcal isolates were used to obtain P+ or P++ preparations that exhibited differences in colony opacity after as few passages in vitro as possible. For all of the strains and colony forms shown in Fig. 2, homogeneous transparent colony preparations were obtained and utilized. For some strains the maximum colony opacity that could be obtained during four to five passages was an intermediate opaque/transparent form, whereas for others very opaque colony preparations were readily obtained. Comparative SDS-PAGE for six such isogenic pairs is shown in Fig. 2. The findings from study of the recently isolated strains are summarized as follows. (i) The apparent molecular weight (M, Fig. 2) for the MOMP varied among the 22 recent isolates in the range of 32 to 34.5K. The finest discrimination in molecular weight was approximately 0.5K, which defined the MOMP size categories. A summary of the MOMP molecular weights found is given in Table 1. (ii) The MOMPs for all colony opacity variants derived from a common parent or found within cultures from a single isolate had the same molecular weights (compare A_1 and A_2, B_1 and B_2, etc., in Fig. 2). (iii) All transparent colony preparations had relatively simple cell wall protein patterns, with the major protein being the most heavily staining moiety of these gonococci (lanes A_2, C_2, etc., Fig. 2). (iv) For each isogenic pair, the opaque (or opaque/transparent) colony form exhibited one or more protein bands not visualized in the corresponding transparent colony preparation (compare A_1 and A_2, B_1 and B_2, etc.). The apparent molecular weights of the protein bands seen only in the opaque colony preparations

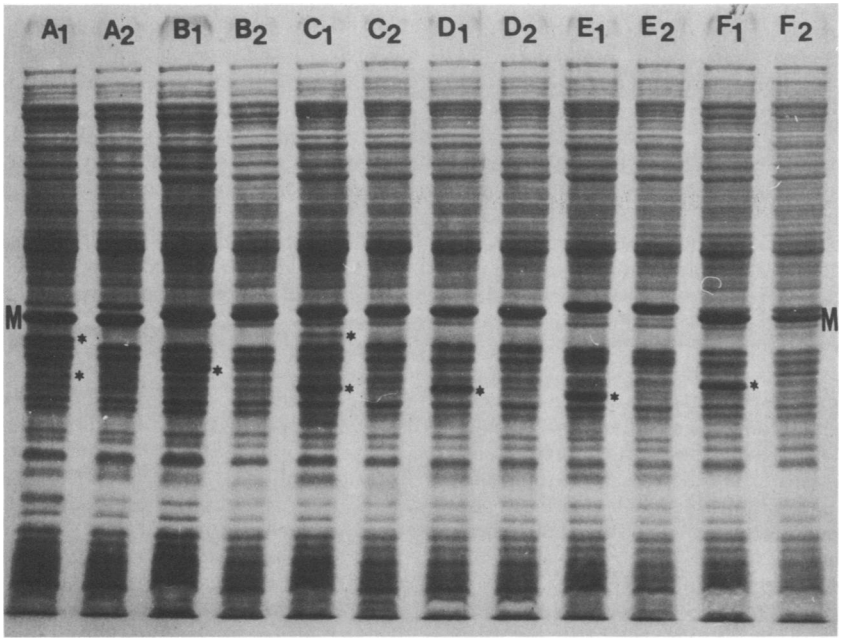


FIG. 2. *Paired transparent and opaque colony preparations were obtained through selective passage of randomly selected clinical isolates, six of which are shown in this gel. For each isolate depicted (A, B, C, etc.), the isogenic opaque (A_1, B_1, C_2, etc.) and transparent (A_2, B_2, C_2, etc.) colony-forming gonococci were compared by SDS-PAGE carried out on SDS-solubilized whole gonococci. Note the similarity in apparent molecular weights (M) for the MOMPs in both members of each isolate (A_1 versus A_2, etc.). Protein bands found only in the opaque colony preparations or more prominent in the same are noted with an *.*

TABLE 1. *Protein bands found in opaque colony forms derived from 22 recent clinical gonococcal isolates*

Mol wt (K) of MOMP[a]	Mol wt (K) of protein in opaque colony[a]
32 (4)	25 (1) 26 (1) 26.5 (2)
32.5 (4)	25 (1) 26 (1) 26.5 (2)
33 (2)	25 (1) 26.5 (1)
33.5 (4)	24 (2) 24.5 (1) 25.5 (1)
34 (3)	25 (1) 25.5 (1) 26.5 (1)
34.5 (5)	23.5 (1) 25 (3) 25.5 (1)

[a] Number of isolates showing band of specified molecular weight noted in parentheses.

varied from isolate to isolate, but the majority were in the 24K- to 26.5K-molecular-weight range. The molecular weights of these proteins were independent of the associated MOMPs (Table 1).

Protein patterns in opaque and transparent colony forms of strain F62. Several colony preparations of pilated (P+ or P++) gonococci from strain F62 were compared by SDS-PAGE. Each of the opaque colony preparations was matched with a transparent form that was similar in passage history and pilation. These were then radiolabeled, incubated with trypsin or buffer, and subjected to SDS-PAGE, and autoradiograms were obtained. The results (Fig. 3), can be summarized as follows. (i) The predominant ^{125}I-labeled band in each transparent colony preparation is the 34K MOMP (lanes B, D, F, and H). The intensity of this band shows little apparent reduction after trypsin treatment of the intact, ^{125}I-labeled transparent colony forms. (ii) Each opaque colony preparation (lanes A, C, E, and G) has a heavily emitting band of 24.5K molecular weight in addition to their 34K MOMP. The intensity of this 24.5K band may equal or exceed that of the 34K band. The intensity of the 24.5K band is markedly reduced in opaque colony preparations incubated with trypsin. The radioemitting intensity of the 34K MOMP is also noticeably reduced by incubation of these opaque colony forms with trypsin before SDS-PAGE. (iii) ^{125}I-labeled bands of 28K, 26.5K, and 26K are variably present, in addition to 24.5K and 34K bands, in these F62 opaque colony preparations. This variation occurred in the different colony preparations whose color/opacity characteristics were identical or very similar by casual examination. The diversity in protein patterns among several opaque colony preparations from a single strain prompted the following comparisons.

A number of colony forms of strain F62 were passaged and, at one point, several P++ colonies were chosen because they exhibited slight differences in the following characteristics: color, opacity, scalloping of colony edge, and clumping of a suspension of the organisms in PBS. Eight homogeneous preparations were obtained by passage of selected colonies, scored for their colonial characteristics, radioiodinated, solubilized in SDS, arranged in the apparent order of decreasing intergonococcal aggregation, and subjected to SDS-PAGE (Fig. 4). It was assumed that the highest degrees of aggregation were expressed as colonies with less than maximal colony coloration, marked but not maximal colony opacity, colony edge scalloping, and pronounced clumping in PBS. The rationale for these assumptions is found in a model described previously (19). Figure 4 shows that the two preparations (lanes A and B) with the highest apparent degree of gonococcal aggregation exhibited more protein bands than did those showing intermediate degrees of intergonococcal adhesions (lanes C to G). Only those colony preparations containing the 24.5K band showed visible clumping in PBS (lanes A to F versus G and H); clumping was seemingly greater when protein bands in the 25.5 to 26.5K range were present in addition to the 24.5K moiety (lanes A and B versus C to F). The transparent/opaque colony (lane G) preparation, which was intermediate in color and exhibited no colony edge scalloping or clumping in PBS, showed the absence of the 24.5K band but had a 26.5K protein not seen in the transparent colony preparation (lane H).

Effect of trypsin treatment on protein and autoradiographic banding patterns of opaque and transparent/opaque gonococci. F62 opaque and transparent/opaque colony preparations that were equivalent (P+) in their pilation were radioiodinated under identical conditions. These ^{125}I-labeled gonococci were incubated with several concentrations of trypsin; Table 2 shows the radiolabel solubilized from these opaque and transparent/opaque forms. As previously noted (19), considerably more ^{125}I is removed from opaque organisms than from iso-

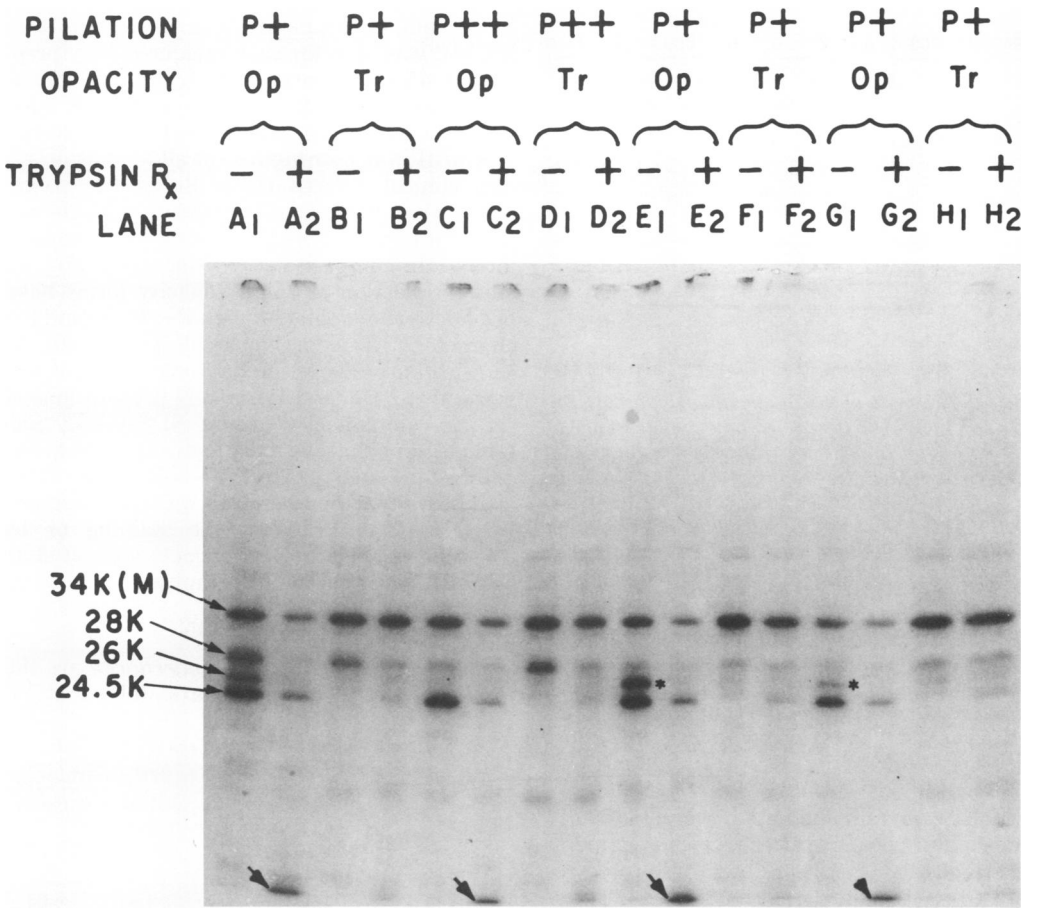


FIG. 3. *Opaque and transparent colony preparations were matched for pilation and passage history, radioiodinated, incubated in buffer or trypsin, lysed in SDS, and subjected to SDS-PAGE, and an autoradiogram was obtained from the gel. Both the buffer control (lanes A_1, B_1, C_1, etc.) and the trypsin-treated organisms (lanes A_2, B_2, C_2, etc.) are shown. Colony forms are as follows: A, E, and G = P+ opaque (Op); B, F, and H = P+ transparent (Tr); C = P++ Op; D = P++ Tr. A and B have a similar passage history as do C and D, E and F, and G and H. Note the similarities in overall banding patterns for all of the transparent colony forms (B_1, D_1, F_1, H_1). Note the prominent ^{125}I-labeled 24.5K band in each opaque colony preparation not exposed to trypsin (A_1, C_1, E_1, G_1). Three of the opaque colony preparations (A_1, E_1, G_1) exhibit additional bands (*) (26 or 26.5K) not found in transparent colony preparations. The latter bands are not seen after trypsin treatment (A_2, E_2, G_2), and their 34K MOMP and 24.5K protein bands are also reduced in autoradiographic intensity for all of the opaque colony forms (A_2, C_2, E_2, G_2). ^{125}I-labeled, small-molecular-weight moieties are found at or near the ion front in trypsin-treated opaque colony forms (arrows in A_2, C_2, E_2, G_2). Little change in banding patterns of transparent colony forms is seen after incubation with trypsin (B_2, D_2, F_2, H_2).*

genic transparent or transparent/opaque colony forms. SDS-PAGE and autoradiography were obtained for these same trypsin-treated gonococcal specimens (Fig. 5 and 6) and demonstrated the following several points. (i) The major bands, as judged by staining or autoradiographic intensity, were the MOMP and one or more bands in the 24 to 30K region. The 34K MOMP was clearly the most prominent band in the transparent/opaque preparations both by Coomassie brilliant blue staining and by autoradiography (Fig. 5). In the opaque variant (Fig. 6), the 24.5K band was quantitatively equivalent to the 34K MOMP in its autoradiographic appearance. (ii) In the P+ transparent/opaque preparation, the intensity of the radioiodinated 26.5K band was reduced in the stained gel and by autoradiography after treatment with trypsin. Other changes in the banding profile were not obvious by examination of the stained gel or its densitometric scan (Fig. 5). (iii) In the P+ opaque preparation, 26 and 24.5K bands, both

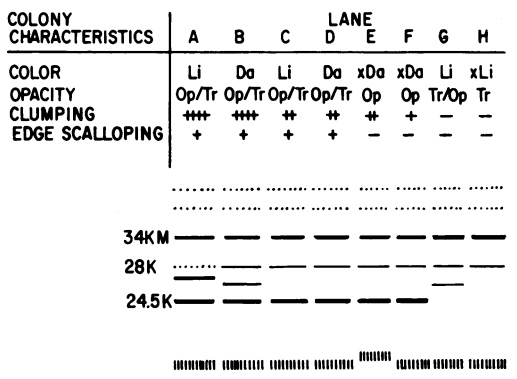


FIG. 4. *Diagram of the autoradiogram after SDS-PAGE of several different colony preparations of strain F62. The colony characteristics are noted on the figure and described in the text. All opaque (Op) and opaque/transparent (Op/Tr) colony preparations (lanes A to F) have a 24.5K band that is radioiodinated. This band is absent from the transparent/opaque (Tr/Op) and transparent (Tr) colony forms (lanes G and H). All colony preparations have MOMPs (34K) that appear identical, and all have a faintly labeled 28K band. Note that those organisms that exhibit marked (++++) clumping in liquid medium (lanes A and B) exhibit a ^{125}I-labeled protein band (26.5 and 26K, respectively) in addition to the 24.5K band. The Tr/Op preparation (lane G) also exhibits the 26K band.*

of which were radioiodinated, were markedly diminished in peak size after trypsin treatment. It appeared that the 24.5K band was more resistant to trypsin than was the 26K one. There was also an apparent reduction in staining intensity of the MOMP of opaque colony forms at the highest trypsin-TPCK concentration used in this experiment. Use of higher concentrations of trypsin resulted in protein pattern changes such as those shown in Fig. 3 for selected colony color/opacity forms. Easily discerned reduction in the audoradiographic intensity of the 34K MOMP was seen for each opaque colony preparation after incubation with trypsin; no apparent analogous effect of trypsin on the 34K band was noted for light, transparent colony forms.

Additional comments. In all studies on recent clinical isolates, either P+ or P++ organisms were utilized, and organisms with the exact same pilation were always used when making interstrain comparisons. Within strain F62, an entire collection of colony opacity forms have been derived for colonies with P+, P++, and P− characteristics, as previously described (20). As shown in a previous, preliminary report of this work, P+, P++, and P− colony preparations of similar color/opacity exhibit SDS-PAGE protein patterns that are indistinguishable from one another in bands in the 24 to 30K range. Thus, it appears that pilus protein was not discerned by Coomassie brilliant blue staining or by ^{125}iodination of whole gonococci subjected to SDS-PAGE and autoradiography.

DISCUSSION

Cell wall proteins of *N. gonorrhoeae* are the topics of several recent reports. Johnston and

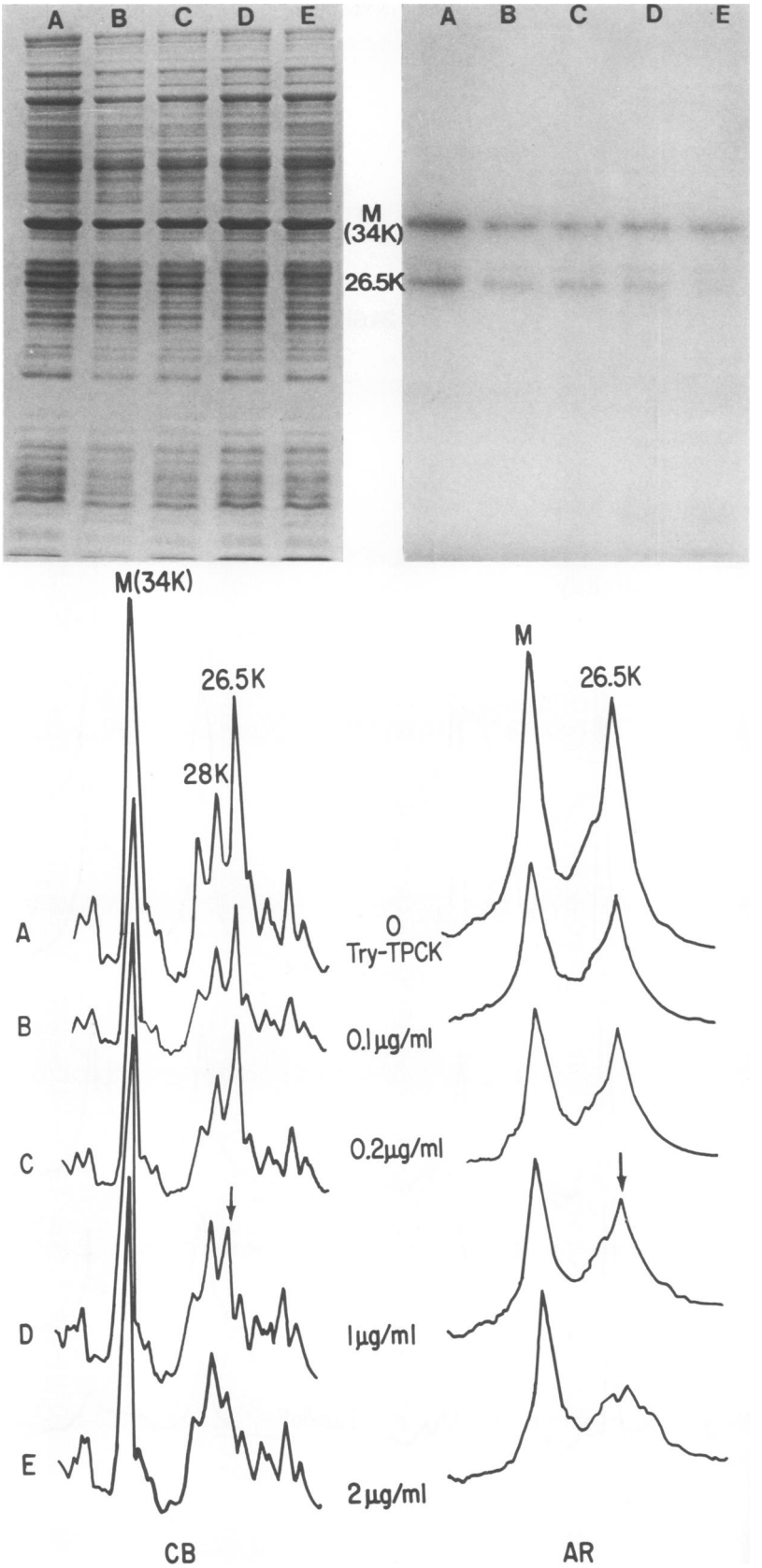
TABLE 2. *Effect of trypsin on ^{125}I-labeled gonococcal color/opacity colony forms*

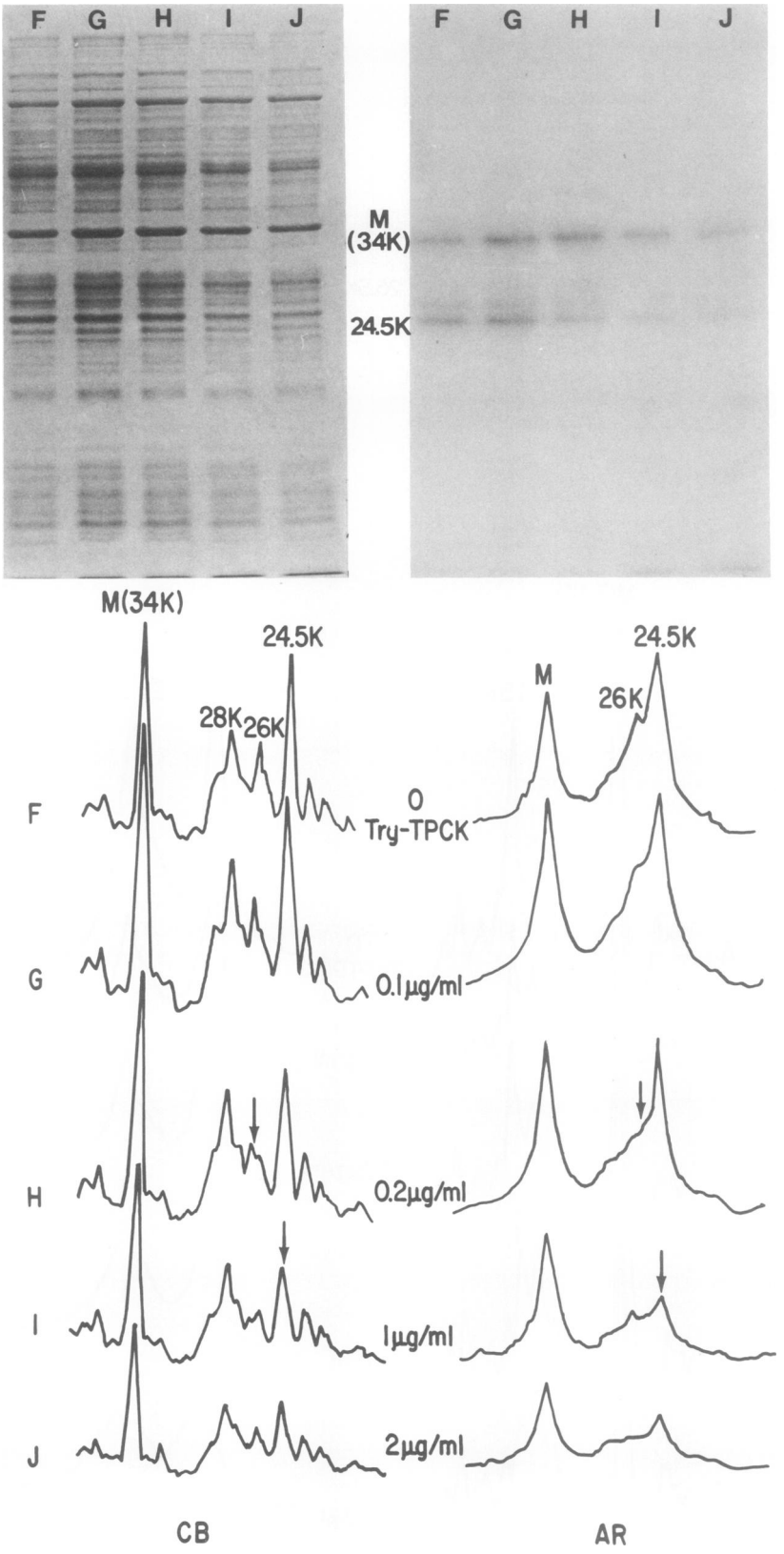
Colony form[a]	^{125}I loss (%) on incubation with trypsin at final concn (μg/ml) of:			
	0.1	0.2	1	2
F62 P+ Tr/Op	1.91	2.75	4.68	6.04
F62 P+ Op	3.04	3.75	9.62	14.42

[a] Tr/Op, Transparent/opaque; Op, opaque.

[b] Percent loss calculated by subtracting the percent loss from control utilizing same transparent/opaque organism suspended in Tris-$CaCl_2$ without trypsin and processed as trypsin-containing specimens.

FIG. 5 and 6. *Gels and autoradiograms of F62 P++ transparent/opaque (Fig. 5) and F62 P++ opaque (Fig. 6) colony preparations that have been radioiodinated and incubated with trypsin at varying concentrations. The whole gonococci were lysed and subjected to SDS-PAGE. The resultant Coomassie brilliant blue-stained gels (upper left panels), the scans of center proteins of the stained gels (CB, lower panels), autoradiograms (upper right panels), and the scans of central portions of the autoradiograms (AR, lower panels) are shown. In the stained gels and autoradiograms, the buffer controls are at the left of each gel (Fig. 5, lane A; Fig. 6, lane F), and the other lanes represent organisms subjected to increasing amounts of trypsin, as noted in the lower panels whose scans correspond to the lane notations in the upper panels. In the transparent/opaque colony preparation shown in Fig. 5, note the ^{125}I labeling of the MOMP M(34K) and of the 26.5K band. The 26.5K component exhibits discernible reduction (arrows, lower panel) in the preparation incubated with 1 μg of trypsin-TPCK (D lanes) per ml. In Fig. 6 of the P++ opaque colony preparation, 26 and 24.5K bands show radioiodination in addition to the MOMP M(34K). The intensity of the 24.5K band by autoradiography appears equal to that of the M(34K) moiety. Note the reductions in peak size of the 26K band (H lanes) on exposure to 0.2 μg of trypsin-TPCK per ml. The band is more markedly reduced at higher trypsin concentrations. Reduction in the 24.5K band is clearly seen at 1 μg (I lanes) and 2 μg (J lanes) of trypsin per ml. There is also an apparent diminution of the intensity of the M(34K) band at the highest concentration of trypsin used (J lanes). A similar reduction in intensity of the M(34K) band is not seen in the transparent/opaque preparation (Fig. 5). These are the same organisms used in the experiment summarized in Table 2.*


A
B
C
D
E
M
(34K)
26.5K
M(34K)
26.5K
28K
M
26.5K
A
0
Try-TPCK
B
0.1μg/ml
C
0.2μg/ml
D
1μg/ml
E
2μg/ml
CB
AR


F G H I J
F G H I J
M
(34K)
24.5K
M(34K)
24.5K
28K
26K
M
26K
24.5K
F
0
Try-TPCK
G
0.1μg/ml
H
0.2μg/ml
I
1μg/ml
J
2μg/ml
CB
AR

Gotschlich (7) have studied isolated cell walls to describe the general properties and proteins of cell wall outer membranes. The dominant outer membrane proteins identified in that report have been subsequently studied as possible serotyping antigens (8). That study demonstrates heterogeneity in the apparent sizes of major outer membranes from strain to strain and also notes the existence of additional minor proteins in the gonococcal cell wall outer membrane. Heckels demonstrated electrophoretic mobility changes in this minor protein (his protein II or II*) depending on the solubilization temperatures used. He also demonstrated that major and minor proteins can be separated from one another by disruption of the cell membrane with deoxycholate and column chromatography (3).

Outer membrane protein differences have been suggested as causally related to differences in the optical properties among gonococcal colonies (19). It has been noted that colony color and opacity characteristics mirror differences in the intercellular aggregation of the gonococci comprising the colonies. In general, the darker, more opaque colonies are made up of highly aggregated gonococci; light, transparent (nonopaque) colonies consist of organisms dispersed as diplococci units that are not adherent to one another. It has also been shown that the highly aggregated organisms are more sensitive to killing and to solubilization of lactoperoxidase-catalyzed, ^{125}I-labeled components from their surfaces by trypsin than are the nonaggregated, transparent colony forms.

In the present studies, gonococci from colonies with varying color and opacity characteristics were compared by SDS-PAGE carried out on whole, lysed organisms. These gel comparisons showed that all transparent colony forms had simple cell wall protein profiles which were all very similar except for differences in the apparent molecular weights of their MOMPs. Opaque colony-forming gonococci had more complex banding patterns and appeared to have one or more prominent proteins not found in isogenic, transparent colony preparations. The apparent size of these colony opacity-associated proteins varied among the isolates and strains examined from 24 to 30K molecular weight. Except for the presence of these additional proteins, opaque colony preparation SDS-PAGE patterns were identical to those of isogenic, transparent colony-forming organisms. Because the additional proteins were readily radioiodinated by the lactoperoxidase-H_2O_2 technique used to label whole, intact gonococci and because the proteins were susceptible to hydrolysis by trypsin, it appeared that their presence was expressed on the outer surface of the gonococcal cell wall. The occurrence of colony opacity-associated proteins was independent of pilation or nonpilation of gonococci, and P+, P++, and P− colonies with dark, opaque or light, transparent characteristics could be identified. However, it appeared that both pili and the colony opacity-associated cell wall proteins contributed to intergonococcal aggregation. If P++ and P− gonococcal preparations of identical cell wall protein patterns were compared, the former (P++) colonies exhibited more edge scalloping, friability, and clumping in liquid medium than did the P− colonies.

No differences in protein banding patterns attributable to pili were seen when P++, P+, and P− colonies were compared by Coomassie brilliant blue staining and autoradiography of SDS-PAGE gels. It is not clear whether this was due to loss of pili during specimen preparation or relative lack of Coomassie brilliant blue staining, fixation of pilus proteins in such gels, and/or insusceptibility of these proteins to ^{125}iodination by the lactoperoxidase method.

The increased susceptibility to killing by trypsin for opaque forms appeared to correlate with hydrolysis of cell wall proteins associated with colony opacity. These 24.5 to 26.5K moieties seemed more liable to the action of trypsin than did the MOMP. It seems likely that the liability of these proteins to tryptic digestions was due to their very superficial location on the gonococcal surface. The MOMP of opaque colony forms was more readily hydrolyzed by trypsin than the apparently identical (at least by molecular weight) protein of isogenic, transparent colony-forming gonococci. It seems possible that hydrolysis of the opacity-associated proteins might perturb the gonococcal cell wall outer membrane proteins. An alternative explanation might be that the MOMP and opacity-associated proteins are both portions of a heteropolymeric moiety and that hydrolysis of the more superficial portion of the polymer increases the susceptibility of the other, more deeply situated MOMP. The latter seems unlikely from the findings of Heckels (3) and my own observation that the opacity-associated proteins can be removed from isolated cell wall outer membranes which retain the MOMP by treatment with deoxycholate. In this context it is interesting that the presence of another gonococcal cell surface protein that is correlated with attachment of gonococci to human neutrophils does not render the major outer membrane more susceptible to trypsin (unpublished data). That difference between leukocyte-associated protein(s) and colony opacity-associated proteins suggested either that the former are more superficially located, that the former are parts of different cell wall protein polymers, or that hydrolysis of leukocyte-associated pro-

tein does not markedly perturb the gonococcal cell wall outer membrane.

The proteins found in the cell wall of the various colony color and opacity forms of gonococci are of interest from several standpoints. First, they may be useful in deciphering the organization of the organism's cell wall. Second, they may provide clues about pathogenic mechanisms involved in the production of gonorrheal infections. This follows from the demonstration that opaque colonies predominate in cultures from males, whereas transparent colonies are more characteristic of cultures from females (4). Clinical isolates from females consist almost exclusively of transparent colonies if the patients are menstruating or are near menstruation. Several cultures from women infected with β-lactamase-producing gonococci revealed differences in colony opacity and opacity-associated proteins; all of the isolates had MOMPs of identical molecular weights and all produced β-lactamase (J. F. James and J. Swanson, unpublished data). These findings suggest, as previously proposed, that host factors may be selective for one or another colony opacity forms in natural infections. Third, the antigenicity of opacity-associated proteins, although currently unknown, is intriguing because of the apparent surface location and molecular weight diversity of these proteins within a strain and among strains.

ACKNOWLEDGMENTS

My thanks to Ethel S. Eldredge, Betty Jean Peterson, and Patty Clark for help in preparing this paper.

This work was supported by Public Health Service grant AI-11073 from the National Institute of Allergy and Infectious Diseases.

LITERATURE CITED

1. **Brown, W. J., and S. J. Kraus.** 1974. Gonococcal colony types. J. Am. Med. Assoc. **228**:862–863.
2. **Dienna, B. B., B. Ryan, F. E. Ashton, and R. Wallace.** 1974. Colonial morphology of *Neisseria gonorrheae* (letter to the editor) J. Am. Med. Assoc. **229**:1422.
3. **Heckels, J. E.** 1977. The surface properties of *Neisseria gonorrheae*: isolation of the major components of the outer membrane. J. Gen. Microbiol. **99**:333–341.
4. **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.
5. **Jephcott, A. E., and A. Reyn.** 1971. *Neisseria gonorrhoeae.* Colony variation I. Acta Pathol. Microbiol. Scand. Sect. B **79**:609–614.
6. **Jephcott, A. E., A. Reyn, and A. Birch-Andersen.** 1971. *Neisseria gonorrhoeae.* III. Demonstration of presumed appendages of cells from different colony types. Acta Pathol. Microbiol. Scand. Sect. B **79**:437–439.
7. **Johnston, K. H., and E. C. Gotschlich.** 1974. Isolation and characterization of the outer membrane of *Neisseria gonorrhoeae.* J. Bacteriol. **119**:250–257.
8. **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.
9. **Kellogg, D. S., I. R. Cohen, L. C. Norins, A. L. Schroeter, and G. Reissig.** 1968. *Neisseria gonorrhoeae.* II. Colonial variation and pathogenicity during 35 months in vitro. J. Bacteriol. **96**:596–605.
10. **Kellogg, D. S., W. L. Peacock, W. E. Deacon, L. Brown, and C. I. Pirkle.** 1963. *Neisseria gonorrhoeae.* I. Virulence genetically linked to clonal variation. J. Bacteriol. **50**:1274–1279.
11. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
12. **Marchalonis, J. J., R. E. Cone, and V. Santer.** 1971. Enzymic iodination. A probe for accessible surface proteins of normal and neoplastic lymphocytes. Biochem. J. **124**:921–927.
13. **Morton, H. E., and J. Shoemaker.** 1945. The identification of *Neisseria gonorrhoeae* by means of bacterial variation and the detection of small colony forms in clinical material. J. Bacteriol. **50**:585–587.
14. **Penn, C. E., D. R. Veale, and H. Smith.** 1977. Selection from gonococci grown *in vitro* of a colony type with some virulence properties of organisms adapted *in vivo.* J. Gen. Microbiol. **100**:147–158.
15. **Raven, C.** 1934. Dissociation of the gonococcus. J. Infect. Dis. **55**:328–339.
16. **Reyn, A., A. E. Jephcott, and H. Ravn.** 1971. *Neisseria gonorrhoeae.* Colony variation II. Acta Pathol. Microbiol. Scand. Sect. B **79**:435–446.
17. **Sparling, P. F., and A. R. Yobs.** 1967. Colonial morphology of *Neisseria gonorrhoeae* isolated from males and females. J. Bacteriol. **93**:513.
18. **Swanson, J.** 1977. Surface components associated with gonococcal-cell interactions, p. 370–401. *In* R. B. Roberts (ed.), The gonococcus. John Wiley and Sons, Inc., New York.
19. **Swanson, J.** 1978. Studies on gonococcus infection. XII. Colony color and opacity variants of gonococci. Infect. Immun. **19**:320–331.
20. **Swanson, J., S. J. Kraus, and E. C. Gotschlich.** 1971. Studies on gonococcus infection. I. Pili and zones of adhesion: their relation to gonococcal growth patterns. J. Exp. Med. **134**:886–905.
21. **Thayer, J. D., and J. E. Martin.** 1964. A selective medium for the cultivation of *N. gonorrhoeae* and *N. meningitides.* Public Health Rep. **79**:49–58.