

## STUDIES ON GONOCOCCUS INFECTION

### II. FREEZE-FRACTURE, FREEZE-ETCH STUDIES ON GONOCOCCI\*

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*Neisseria gonorrhoeae* is certainly one of the commonest pathogens for man. Despite the long history and the ubiquity of gonorrhea, relatively little is actually known about the pathobiology of the causative organism or about the relationships that exist between the infecting gonococcus and the infected host. Several recent studies have begun to provide clues as to the importance of the gonococcus surface as a pathogenetic determiner in the interaction between microorganism and host. Virulence of gonococci in man (1, 2) has been shown to correlate with formation of colonies with characteristic morphologies. The type 1 and type 2 colonies described by Kellog et al. (1) contain virulent gonococci, whereas avirulent gonococci form type 3 or type 4 colonies on agar medium. More recently it has been found that organisms from type 1 and type 2 colonies bear pili, whereas gonococci from type 3 and type 4 colonies do not have pili (3, 4). The apparent association between virulence and presence of pili is intriguing. Additional studies have demonstrated anti-pilus antibody production in humans and chimpanzees infected by gonococci (5). Preliminary investigations on interactions between gonococci and tissue culture cells suggest additional mechanisms by which gonococci play a pathogenetic role.<sup>1</sup> The crux of all the above mentioned observations is that the exterior surface of the gonococcus is intimately involved, through one or several mechanisms, in causation of acute gonorrhea.

The present study employs the technique of freeze-cleavage, freeze-etching preparation of pilus-bearing gonococci for study by electron microscopy to document more fully the morphological characteristics of the gonococcal surface.

#### *Materials and Methods*

*Strains of Neisseria gonorrhoeae.*—Several strains were utilized in this study and included both recently isolated strains (MS-11 and MS-12) obtained in the outpatient clinic and two strains (F62 and 2868) which were supplied by Dr. Douglas S. Kellogg of the Venereal Disease Research Laboratory, Atlanta. Cultivation, identification, storage, and transfer of

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colony types 1 and 2 from these strains were carried out as previously described (4). Organisms were grown either on solid medium (GC agar base supplemented with Iso-Vitalex; Baltimore Biological Laboratories, Baltimore, Md.) after selective transfer of a single colony of desired type and incubation for 16–18 hr at 37°C in a candle extinction jar or in modified Frantz medium (6) supplemented with 0.4% yeast extract (Difco Laboratories, Detroit, Mich.) after inoculation of the liquid medium with several colonies from agar medium and incubation in a rotary shaking water bath at 37°C for 4–6 hr.

*Electron Microscopy.*—

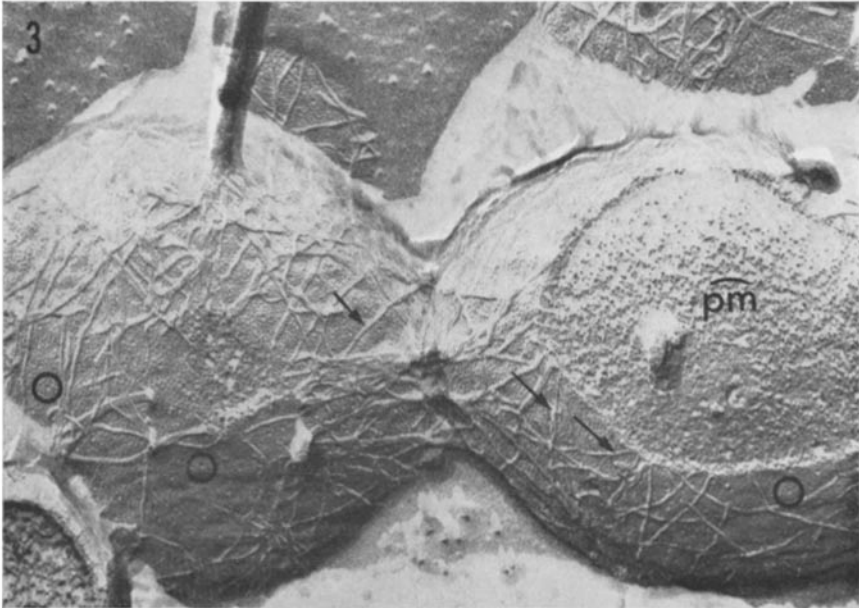
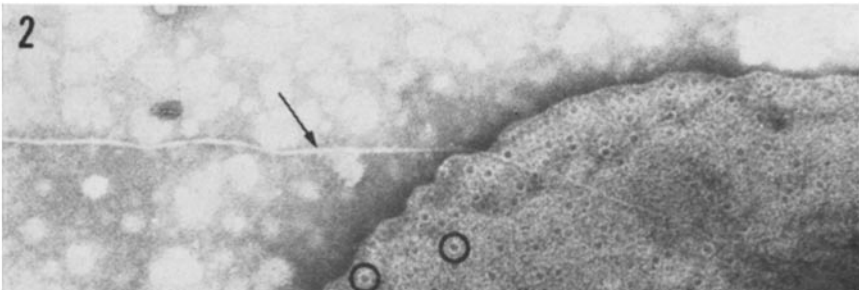
*Thin sectioning and negative staining:* These procedures have been described in detail elsewhere (4).

*Freeze-cleavage, freeze-etching:* Organisms from both agar and liquid cultures of type 2 gonococci were processed by freeze-cleavage, freeze-etching either as unfixed or as fixed (2% glutaraldehyde in 0.1 M sodium cacodylate, pH 6.9, 2–20 M, room temperature) specimens. For preparation in the absence of glycerol, the organisms were centrifuged and resuspended in a small amount of buffer (0.1 M sodium cacodylate, pH 7.0) or in water before freezing in Freon. Glycerol treatment was carried out by suspending fixed or unfixed gonococci in 25% glycerol in 0.1 M sodium cacodylate for 1 hr at 4°C before freezing. After immersion in Freon, the specimens were transferred to liquid nitrogen and stored briefly before completion of the preparatory process. After transfer to a Balzers 360 M apparatus (Balzers High Vacuum Corp., Santa Ana, Calif.), the specimens were fractured and etched (2–3 min, –100°C), and replicas formed as described in the Balzers Instruction Manual (7). Replicas were floated on Clorox and cleaned for 3 hr after which they were washed with distilled water. The cleaned, washed replicas were mounted on naked copper grids and were studied with an AEI EM801 microscope (AEI Scientific Apparatus Inc., White Plains, N. Y.) equipped with tilting specimen holder (20°) and operating at 80 kv. Images were recorded on Kodak Electron Microscope Film (Eastman Kodak, Rochester, N. Y.) and were printed without reversal.

## RESULTS

*Review of Gonococcus Structure Seen in Thin Sections and by Negative Staining.*—Gonococci observed in thin sections exhibit surface coverings typical of Gram-negative bacteria (Fig. 1). The cell wall consists of two parts: an outer membrane and an inner, single dense lamina. The cell wall outer membrane has a trilaminar profile, is 75–85 Å thick, and follows an undulating course on the exterior of the organism. The single dense lamina mimics the contour of the protoplast membrane, is extremely electron opaque, and is approximately 60 Å in thickness. Beneath the cell wall is the protoplast or cytoplasmic membrane which is similar in lamination and thickness to the cell wall outer membrane. Individual and aggregated pili can often be visualized external to the cell. Individual pili are usually difficult to recognize in thin sections but can be seen occasionally projecting from the cell exterior (Fig. 1).

Negative staining allows visualization of both the exterior of the gonococcus and the pili that radiate from its surface (Fig. 2). One of the most striking features of the negatively stained surface of the gonococcus is the presence of “holes” in the wall. These focal perforations are suggested by the presence of numerous discrete accumulations of negative stain surrounded by electron-lucent halos. These holes are similar to structures seen in meningococci where they have been localized to the outer membrane of the cell wall (8). Holes are



present on gonococci of all four of Kellogg's colony types examined by negative staining. Pili are observed radiating from the surfaces of colony types 1 and 2 gonococci after negative staining and have been previously described (4). Pili are not well demonstrated on the *en face* viewed surfaces of negatively stained preparations of gonococci and are visualized best as they extend from the peripheries of the organisms. Pili are not found on gonococci derived from type 3 or type 4 colonies.

*Freeze-Cleavage, Freeze-Etching.*—

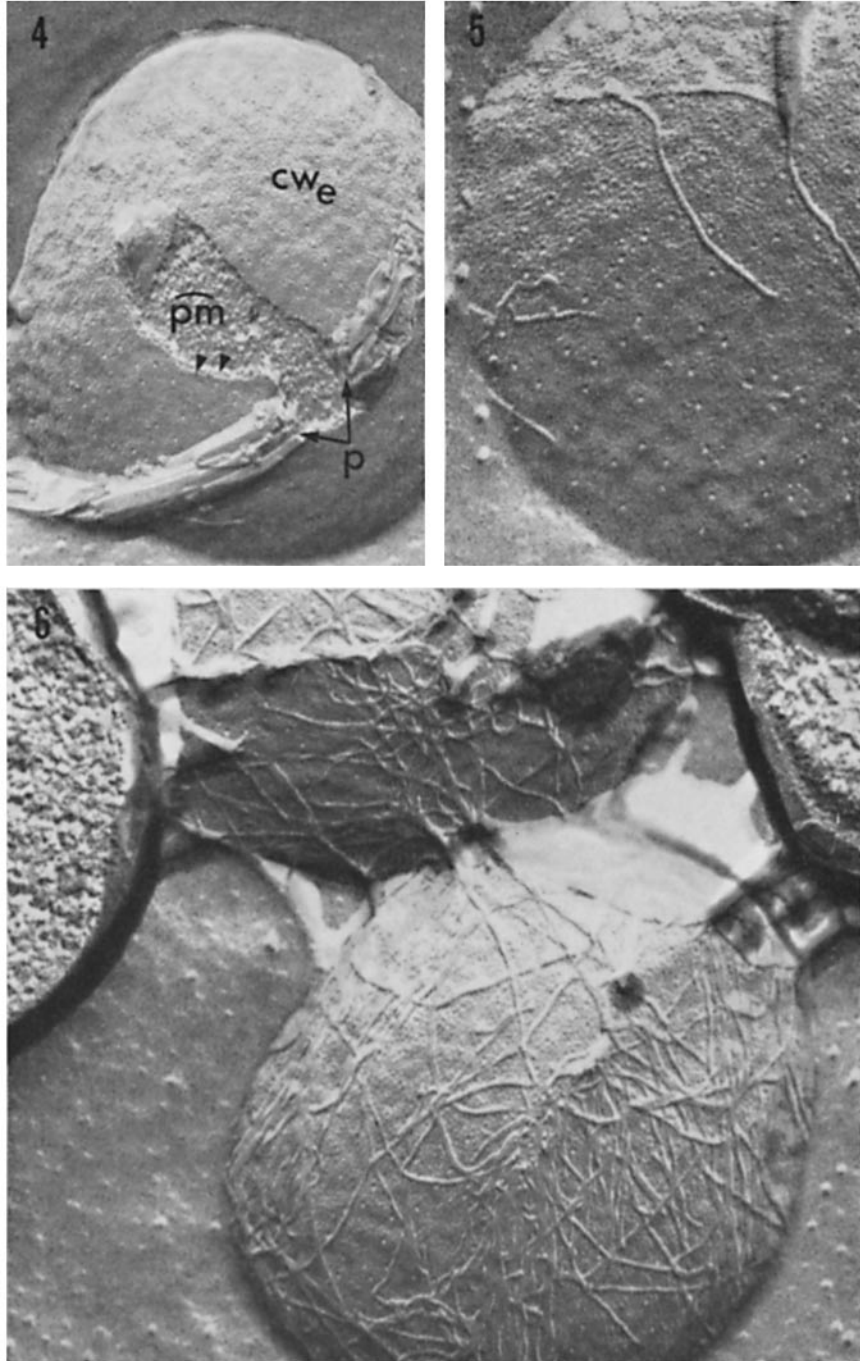
*General comments:* In the preparation of specimens for electron microscopic examination by freeze-fracture, freeze-etching, several procedural steps dictate the morphology in the resultant replica. The incident angle at which the cooled microtome knife strikes the frozen bacterium and the presence of glycerol influence the internal structure that is revealed. When the knife strikes the gonococcus near its equator, the organism is sliced or sectioned and little fracturing occurs. In this instance the surface layers of the gonococcus are cut at a nearly perpendicular angle and little detail of these laminae is observed. Visualization of the spherical bacterial surface coatings is enhanced if the knife strikes the gonococcus near its upper or lower pole. When this occurs, fracture of the frozen specimen follows cleavage planes within the multilaminar cell coverings. The preferential plane of fracture for both bacteria and eukaryotic cells appears to be the middle of the protoplast or cytoplasmic membrane

All the gonococci shown are from type 2 colonies of various strains. Figs. 3–12 which are replicas of freeze-fractured, freeze-etched organisms have been printed without reversal and are mounted so the direction of platinum shadowing is from the top of the plate. Figs. 3–9 are of gonococci prepared in the absence of glycerol, and Figs. 10–12 contain specimens frozen in 25% glycerol.

FIG. 1. In thin sections the laminae of the type 2 gonococcal cell wall are easily observed and consist of an outer membrane (*om*) and a single dense lamina (*dl*) surrounding the protoplast or cytoplasmic membrane (*pm*). Aggregates of pili are found in the intercellular area (\*), and in some instances individual pili (*arrows*) can be found extending from the gonococcal surface. Small blebs (*b*) or evaginations of the cell wall outer membrane may also be present.  $\times 62,500$ .

FIG. 2. Through negative staining with uranyl acetate the gonococcal surface may be seen *en face* in lightly stained organisms. Small accumulations of negative stain surrounded by halos (examples encircled) give the impression of "holes" or pits in the surface of the organism. A pilus (*arrow*) can be seen extending from the surface of the type 2 gonococcus, but is poorly visualized coursing across the exterior *per se*.  $\times 100,000$ .

FIG. 3. These type 2 gonococci, freeze-fractured, freeze-etched in the absence of glycerol, display several planes exposed by the process. The cell on the left has barely been grazed by the microtome knife, whereas the cell in the lower left corner (partially shown) has been transected near its equator. The gonococcus on the right exhibits cleavage-removal of a portion of its cell wall to expose a fracture plane through its split protoplast membrane (*pm*). Etching has exposed additional surface on the organisms; the exterior of each is covered by pili (*arrows*) coursing on their surfaces. Depressions or holes (encircled) are also seen on the etch-exposed exteriors of these gonococci.  $\times 62,500$ .



which is split by such cleavage (9-12). If one views the split membrane from which the outer portion has been removed by the microtome knife, one observes the convex surface of the split membrane ( $\widehat{pm}$ ). When the microtome knife strikes the bacterium near its lower pole, the bulk of the cell contents is scooped out by the process leaving a fragment of the surface laminae behind. In this case the inner half of the split cytoplasmic membrane has been removed by the knife's passage and one views the outer half of the membrane (from its inner aspect): the concave face of the split membrane ( $\widehat{pm}$ ). The influence of glycerol on cleavage planes produced will be shown and described below.

The etching process may uncover portions of the bacterium's exterior due to vacuum sublimation of water from the extracellular space. Thus, one may observe not only the deep portions of the bacterium revealed by cleavage but also the surface of the organism exposed through etching. Etching is markedly reduced when glycerol is present in the freezing mixture; therefore, one deletes glycerol in order to achieve visualization of the gonococcus exterior uncovered by etching.

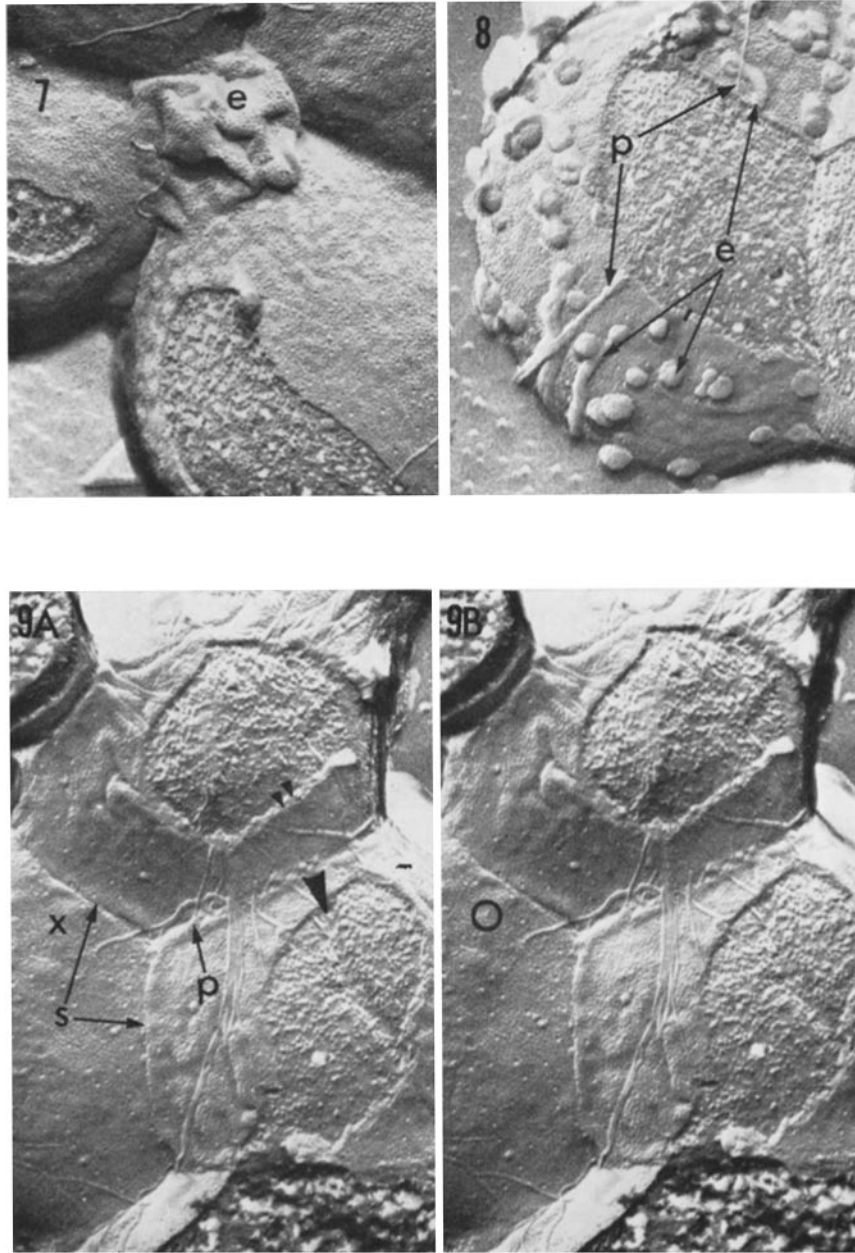
*Gonococci prepared in the absence of glycerol:* The external surface of type 2 gonococci subjected to freeze-etching in the absence of glycerol is extensively exposed and is recognizable by the presence of pili (Figs. 3-9). The pili course along the surface ( $cv_e$ ) of the gonococci in apparent intimate contact with the organism. The number of pili on a particular organism varies from only a few (Fig. 5) to such a large number that the major part of the organism's surface appears to be covered by the pili (Figs. 3 and 6). In some instances crystalloid arrays of pili are seen (Fig. 4). The dimensions and periodicity of surface pili correspond to those found by negative staining.

The external surfaces of gonococci have a slightly wrinkled appearance and a pebbled texture. The subunits that contribute to this pebbled texture are about 75-85 Å in diameter. Also visible on the external surfaces of gonococci are pits or holes similar in diameter to the foci seen by negative staining. The pits reside on the raised portions of the wrinkled gonococcal surface and do not appear to be arranged in a geometric fashion.

Planes beneath the surface are exposed by freeze fracture of many of the gonococci. As previously mentioned, the preferential plane of fracture is within the protoplast membrane. The convex fracture plane ( $\widehat{pm}$ ) is studded with

FIG. 4. The majority of pili on this gonococcus are aggregated into a crystalloid bundle which has been fractured by the freeze-cleavage ( $p$ ). The outermost layer exposed through etching ( $cv_e$ ) has been fractured to reveal the underlying cleavage plane of the protoplast membrane ( $\widehat{pm}$ ). At the edges of this cell wall fracture two distinct laminae or planes are seen (*arrow heads*).  $\times 62,500$ .

FIGS. 5 and 6. The number of pili seen on gonococci varies from only a few (Fig. 5) to so many that the majority of the gonococcus' surface is covered (Fig. 6; also see Fig. 3).  $\times 62,500$ .



FIGS. 7 and 8. Evagination (*e*) of the cell wall are seen in shapes varying from bag-like (Fig. 7) to wart-like or elongated (Fig. 8). The elongated profiles superficially resemble aggregated pili (lower *p*; Fig. 8), but the latter have periodicity not found in the evaginated cell wall.  $\times 62,500$ .

spherical particles whose distribution is somewhat uneven when prepared in the absence of glycerol. The edges of the fractured gonococcus surface reveal two distinct laminae that have been cleaved (Figs. 3, 4, 9). Pili course on the gonococcus exterior to the edges of the fractured cell wall and are discontinuous at that point due to their having been fractured by the freeze-cleavage.

Gonococci often exhibit evaginations of their cell walls that are visible as blebs, wart-like or bag-like protrusions, or elongate structures on the cell surface (Figs. 7, 8). The elongate structures are superficially similar to bundles of pili coursing along the cell's exterior, but the latter have periodicity along their length to distinguish them from the former (Fig. 8).

The exterior margins of adherence zones between cells are well seen (*s*; Fig. 9 A) as linear seams or crevices that mark interfaces between adjacent cells. These seams have been found in greatest abundance in fluid cultures in which the gonococci grew as grossly clumped aggregates in the medium. The cell walls of adjoining organisms appear tightly apposed and pili course from the surface of one gonococcus to the next without interruption. Some pili appear to emanate from the depths of the seams (*p*; Figure 9 A).

Gonococci from type 3 and type 4 colonies have also been studied by this procedure. The cell walls of these organisms (not shown) are identical in all respects, except for the absence of pili, to the cell walls of type 1 and type 2 gonococci.

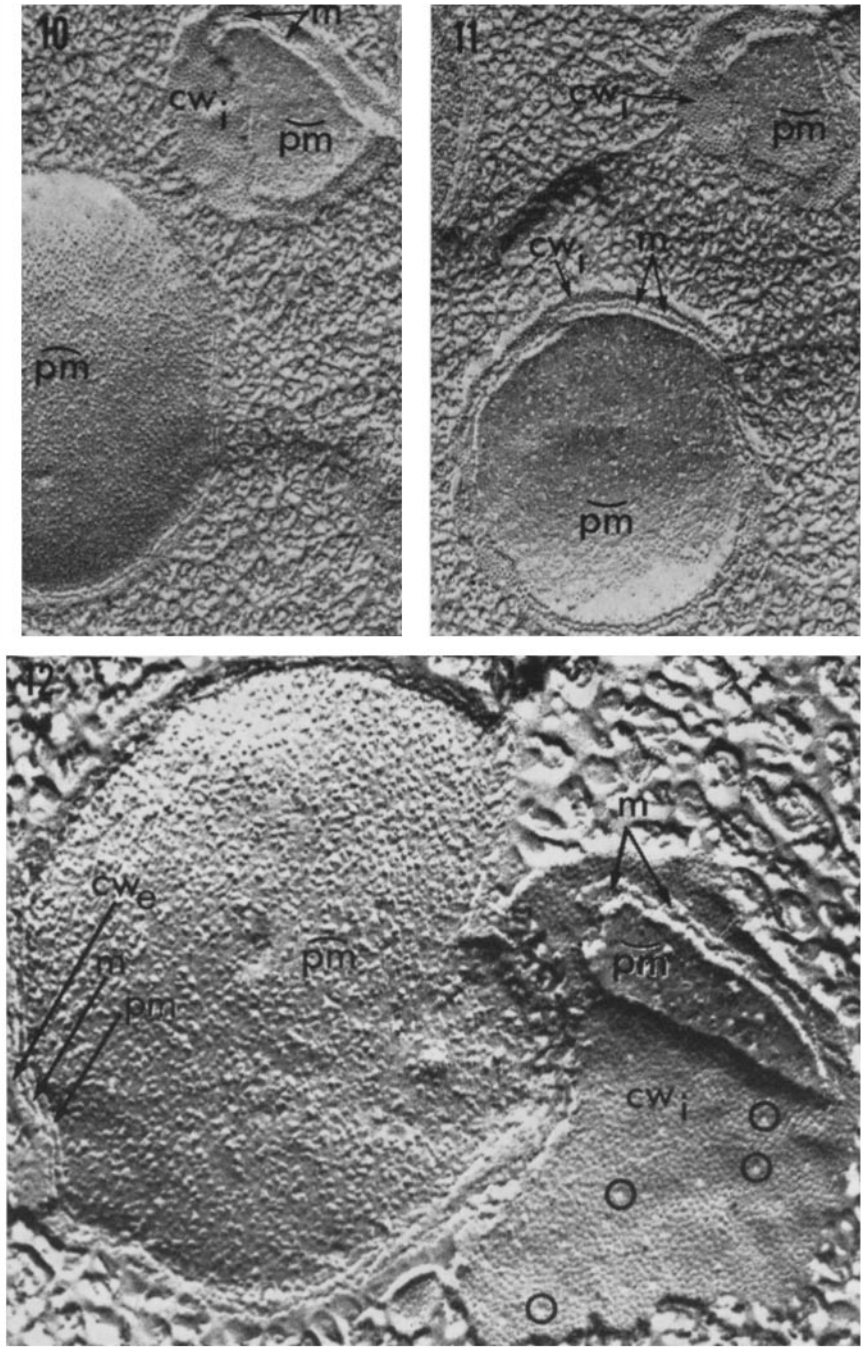
*Gonococci prepared in the presence of glycerol:* Many gonococci freeze-fractured in the presence of glycerol exhibit the same cleavage plane through their protoplast membranes as seen in specimens prepared in the absence of glycerol. When glycerol is in the freezing mixture, this convex face of the plasma membrane appears more heavily studded by spherical particles whose individual identities are discreet (Figs. 10 and 12). The raised contour of an organism whose exposed surface is this convex plasma membrane plane is surrounded by a rim of material representing the outer laminae of the cell wall and outer half of the plasma membrane. Little detail in structure can be discerned in this rim of concentric layers, but at least two and possibly three discreet laminae are present (Figs. 10 and 12).

The predominant concave profile of gonococci freeze-fractured in the presence

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FIG. 9 A and B. This stereo pair is best studied by viewing the left figure (9 A) with the right eye, the right figure (9 B) with the left eye, and fusing the images. Alternative methods include separating the right and left fields of vision with a piece of stiff cardboard extending about 14 inches from the observer's nose to the zone between the paired figures. Fusion of the images from the pair is accomplished when the *X* of the Fig. 9 A is seen within the *O* of Fig. 9 B. Cell wall outer membranes from neighboring cells appear tightly fused at seams (*s*) among the cells. Pili course over the surfaces of the organisms and pass from one organism to the next. In some instances pili appear to emanate from the seams between cells (*p*). At the edges of the fractured cell wall, two distinct laminae or planes are seen (*small arrows*). Also striking is a depressed linear region in the fractured protoplast membrane (*large arrow*) which is probably related to a developing division septum.  $\times 62,500$ .





of glycerol is shown in Fig. 11. The major surface most likely represents the outer half of the split protoplast membrane ( $\overline{pm}$ ) and is relatively smooth. Adherent fragments of the laminae external to this fracture plane are visible near the peripheries of these concave profiles. Two or three separate laminae are present. The most clearly defined layer appears to be the outermost lamina of the cell wall. This lamina has definite subunit structure on its fracture plane and the subunits are identical with those seen on the exterior of the cell wall. Each subunit is round to hexagonal in shape and is 80 Å in diameter (center to center). Also discernible on this layer are pits or holes whose diameters are the same as the pits or holes seen on the exterior of the gonococci. It is presently not clear whether this layer ( $cw_i$ ) is the full thickness or split thickness of the cell wall outer membrane. Between the  $cw_i$  plane and the  $\overline{pm}$  face is an intermediate fracture surface ( $m$ ) which is seen as a thin edge in the preparations studied. This edge may represent either the material observed as a single dense lamina in thin sections or the inner half of the split cell wall outer membrane.

#### DISCUSSION

The importance of bacterial cell wall and surface constituents in determining the pathobiological characteristics of the organisms is well known for a variety of genera including pneumococci, streptococci, staphylococci, and enterobacteria. The roles played by these bacterial surface components extend from determination of interactions with phagocytes to presentation of antigenic substances against which protective antibodies can be formed.

Although our understanding of the pathobiology of *Neisseria gonorrhoeae* is far from complete, evidence suggesting an important role of the gonococcal surface is emerging. This evidence stems from the observations of the virulence colony morphology relationships first found by Kellogg and coworkers (1, 2). Further examination of gonococci derived from Kellogg's colony types revealed a pili:colony morphology relationship suggestive that pili are instru-

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FIGS. 10-12. In the presence of glycerol the predominant cleavage planes are the convex ( $\overline{pm}$ ; Figs. 10 and 12) and the concave ( $\overline{pm}$ ) fracture faces of the protoplast membrane. The surface laminae external to these cleavage planes are seen as thin, concentrically laminated rims at the periphery of each cell. In cells that have been scooped or shelled out by the cleavage (as in Fig. 11), two layers in addition to the outer portion of the protoplast membrane are visible. These represent the cell wall ( $cw_i$ ) and a deeper layer ( $m$ ). The same laminae are visible when small fragments of the surface layers are retained in the specimen after cleavage. In these fragments (Figs. 10-12) the concave plane of the protoplast membrane ( $\overline{pm}$ ), the outer cell wall layer ( $cw_i$ ), and the interposed, middle layer ( $m$ ) are visible. Holes (encircled) are present on this inner fracture plane of the cell wall. The cleavage plane ( $cw_i$ ) of the cell wall has a hexagonal to spherical subunit structure and a slightly wavy contour. In some instances in which the convex plane of the protoplast membrane is visible, three layers of external coverings are also seen ( $cw_e$ ,  $m$ , and  $\overline{pm}$ ) as in Fig. 12. Figs. 10 and 11,  $\times 62,500$ ; Fig. 12,  $\times 100,000$ .

mental as determiners of gonococcus' virulence (4). Although the mechanism through which pili might effect this causal relationship is not fully elucidated, recent studies have shown the importance of pili as antigens that promote an immunological response in patients with gonorrhea (5). Further, preliminary studies in my laboratory have shown striking differences between pilated and nonpilated gonococci in their interactions with eukaryotic cells in vitro. Resolution of the role played by the gonococcus' surface as a determiner of the organism's pathogenicity depends on gaining understanding of the nature of the gonococcus' surface per se.

The cell walls of both untreated meningococci (8) and gonococci (4) have been shown to bear focal collections of negative stain surrounded by electron-lucent halos. These foci are similar in morphology to holes that result from interaction of antibodies and complement with membrane antigens in a variety of cell types (13-15). The ultrastructure of the membranes by negative staining strongly suggests they are indeed holes; however, the possibility remains that the negative stain accumulations represent membrane foci that bind large numbers of stain molecules by virtue of their chemical composition rather than because of their depressed contour. The present freeze-cleavage, freeze-etch studies provide additional evidence that these cell wall foci are actual holes or pits. The holes have been demonstrated not only on the external surface but also in the deeper cleavage planes of the cell wall. It is not clear whether these deeper planes represent the inner aspect of full- or split-thickness cell wall outer membrane. However, it is clear that the holes extend at least partially through the cell wall outer membrane. One might argue that the holes or pits are formed when small foci rich in water or aqueous material undergo accelerated sublimation and are lost from the cell wall during the etching procedure. This possibility cannot be excluded definitely, but it seems unlikely inasmuch as the holes are found in specimens freeze-fractured, freeze-etched in the presence of glycerol, which markedly reduces the extent of sublimation or etching.

A previous study demonstrated the zones of adhesion that are found between adjacent gonococci and are associated with visible clumping of the organisms in fluid medium (4). These zones trap lanthanum nitrate and have dimensions suggesting that they are analogous to the "gap" junctions observed in a variety of eukaryotic cell types (16-18). Gap junctions are further characterized by the presence of close-packed arrays of 60-A diameter particles with 80-A center-to-center spacing on one cleaved plane of the plasma membranes participating in these junctions. The complementary face of the fractured gap junction membrane (also within the membrane) exhibits pits that roughly correspond in complementary fashion to the particles on the other half of the membrane. I have been unable to identify cell wall outer membranes in areas comprising zones of adherence. The preferential cleavage plane through such

multilaminar (four membrane layers and two single dense laminae) zones appears to be the protoplast membranes of the adjacent organisms. Therefore, it is not at present possible to determine whether zones of adhesion are structurally identical with gap junctions. It is perhaps noteworthy, however, that the fracture plane which probably courses through the outer membrane of the gonococcus' cell wall exhibits subunits that are similar to those present in gap junction membranes. In the case of gonococci these subunits do not constitute a specialized region but characterize the entire cell wall outer membrane.

Bacterial pili have previously been demonstrated through negative staining or by formation of heavy metal replicas on dried specimens (19). In both these procedures the pili appear to protrude from the surface of the organisms in radial fashion. The present study of freeze-cleaved, freeze-etched gonococci reveals a markedly different pattern of disposition of pili relative to the bacterium's surface. The pili adhere to and follow the gross and fine contours of the cell wall surface. What might explain the radically different appearances of pili with these different preparative methods? The supporting substrates for negative staining and heavy metal shadowing are relatively hydrophobic surfaces such as Formvar or collodion coated with carbon or glass. Such surfaces might preferentially attract pili which would spread as they adhere and produce the artifact of a radiating appearance. On the other hand, pili extending from the exteriors of gonococci can sometimes be seen in thin sections. This tends to support the concept that the pili radiate from the organism's surface. During freeze-fracture, freeze-etching the organisms are presumably frozen instantaneously in the same form as they exist in a liquid medium. Prefixation with glutaraldehyde might be expected to produce some aggregation of pili, but the identical appearance of surface-adhering pili has been found in both fixed and unfixed specimens. The radiation of pili from the gonococcus' surface might be difficult to appreciate through this technique. Those pili which extended above the bacterium would either be sheared by the freeze-fracture or would be left unsupported by etching and would collapse during that stage of preparation. One cannot exclude the possibility that during etching the pili projecting from the exposed surface of the bacteria collapse as the extracellular supporting matrix is sublimated. The fact that no pili can be found lying on the cleaved planes of the gonococci seems to militate against that explanation.

Interest in the orientation of pili with respect to the cell wall exterior of the gonococcus derives from speculations on the possible role that pili play in interactions between bacteria and eukaryotic cells (4, 20). If the pili were disposed as radially oriented structures, their potential sphere of influence on the surface of the bacterium might be analogous to that of the classical bacterial capsule. If, on the other hand, the pili maintain close adherence to the gonococcus' exterior, their relationship might be more analogous to M-antigen or group-specific polysaccharides of streptococci where the components are integral

components of the wall per se. At present I favor the concept that pili are usually in close contact with the gonococcus' cell wall surface and that radiating pili represent a minor proportion of these cell wall appendages that are present.

#### SUMMARY

Gonococci have been studied by electron microscopy after freeze-cleavage, freeze-etching and the findings correlated with those obtainable through thin sectioning and negative staining. The outer membrane of the cell wall is composed of round to hexagonal subunits 80 A in diameter. This membrane is also punctuated by 80-A holes visible on the exterior of the organism and extending into the substance or through the outer membrane. Pili coursing over the surface of the organisms appear to maintain a close anatomical relationship with the cell wall. In some instances, the surfaces of the organisms are virtually covered by a layer of pili.

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