# *Neisseria* Pili Proteins: Amino-Terminal Amino Acid Sequences and Identification of an Unusual Amino Acid†

Mark A. Hermodson,*,‡ Kirk C. S. Chen, and Thomas M. Buchanan

ABSTRACT: The amino-terminal amino acid sequences of the pili proteins from four antigenically dissimilar strains of *Neisseria gonorrhoeae,* from *Neisseria meningiditis,* and from *Escherichia coli* were determined. Although antibodies raised to the pili protein from a given strain of gonococcus cross-reacted poorly or not at all with each of the other strains tested, the amino-terminal sequences were all identical. The meningococcal protein sequence was also identical with the gonococcal sequence through 29 residues, and this sequence was highly homologous to the sequence of the pili protein of *Moraxella nonliquifaciens* determined by other workers. However, the sequence of the pili protein from *E. coli* showed no similarity to the other sequences. The gonococcal and meningococcal proteins have an unusual amino acid at the amino termini, *N*-methylphenylalanine. In addition, the first 24 residues of these proteins have only two hydrophilic residues (at positions 2 and 5) with the rest being predominantly aliphatic hydrophobic amino acids. The preservation of this highly unusual sequence among five antigenically dissimilar *Neisseria* pili proteins implies a role for the amino-terminal structure in pilus function. The amino terminus may be directly or indirectly (through preservation of tertiary structure) important for the pilus function of facilitating attachment of bacteria to human cells.

Pili were first reported on *Neisseria gonorrhoeae* in 1971 (Jephcott et al., 1971; Swanson et al., 1971). They were found only on gonococci capable of producing human gonorrhea, and nonpiliated organisms were avirulent colony types (Kellogg et al., 1963, 1968). Soon thereafter, piliated organisms were found more virulent than nonpiliated gonococci for chimpanzees (Lucas et al., 1971; Brown et al., 1972) and chick embryos (Buchanan & Gotschlich, 1973; Bumgarner & Finkelstein, 1973). This association of piliation with virulence is thought to be due to the role of pili to facilitate bacterial attachment to cells. Piliated gonococci attach more readily than nonpiliated organisms to red blood cells (Punsalang & Sawyer, 1973; Waitkins, 1974; Koransky et al., 1975; Buchanan & Pearce, 1976), tissue culture cells (Swanson, 1973; Swanson et al., 1975), human vaginal epithelial cells (Mardh & Westrom, 1976), human Fallopian tube mucosa (Ward et al., 1974), and human sperm (James-Holmquest et al., 1974; James et al., 1976). Gonococcal pili were first purified in 1972 (Buchanan et al., 1972, 1973). These and subsequent studies indicated that gonococcal pili are composed of protein of a single repeating subunit with a molecular weight of approximately 18 000 (Ward & Robertson, 1976; Buchanan, 1977). Studies with purified gonococcal pili indicate considerable antigenic heterogeneity among different strains (Buchanan, 1975, 1977; Novotny & Turner, 1975; Buchanan & Pearce, 1976). However, antigenically different gonococcal pili are similar in their attachment to human cells, and a common cell surface receptor for all gonococcal pili has been proposed (Pearce & Buchanan, 1978). Interestingly, gonococcal pili tend to attach end on to cell surfaces, and it has been suggested that only a single end of the isolated pilus contains the attachment moiety (Swanson, 1973; Buchanan, 1977). The chemical nature of the receptor for gonococcal pili is unknown, but the pili of *Escherichia coli* appear to be "lectin-like" and recognize mannose containing cell surface receptors (Ofek et al., 1977). The only reported sequence analysis of pili is an amino-terminal structure for *Moraxella nonliquifaciens* (Frøholm & Sletten, 1977). In the following study, we examined the amino-terminal sequence for four antigenically distinct gonococcal pili (strains B, F62, 33, and 7122), strain 13090 (ATCC) of *Neisseria meningitidis,* and a piliated, nonflagellated strain of *E. coli.* Through these investigations we sought to determine whether the amino-terminal structure of gonococcal pili affects the antigenicity or attachment function of the molecule.

## Materials and Methods

*Bacteria.* The four strains of *N. gonorrhoeae* used to produce gonococcal pili were different as determined by antigenic (Johnston et al., 1976; Wang et al., 1977) and nutritional markers (Catlin, 1973; Carifo & Catlin, 1973; Knapp & Holmes, 1975) (Table I). Three strains (F62, 33, B) were isolated from patients with localized gonococcal urethritis, and the fourth strain (7122) possessed characteristics that are usually present in strains capable of causing disseminated gonococcal infection [extreme penicillin sensitivity (Wiesner et al., 1973) and resistance to killing by normal human serum (Schoolnik et al., 1976)]. The pili purified from these strains were also antigenically dissimilar as demonstrated qualitatively by Ouchterlony immunodiffusion, two-dimensional immunoelectrophoresis, and rocket immunoelectrophoresis (Buchanan & Pearce, 1976; Buchanan, 1977; Pearce & Buchanan, 1978) and quantitatively by radioimmunoassay (Buchanan & Pearce, 1976; Buchanan, 1975) (<3% shared antigenicity).

The strain of *Neisseria meningitidis* used to prepare meningococcal pili was ATCC 13090, a group B meningococcus shown to possess stable piliation not lost with multiple subcultures on artificial media (Devoe & Gilchrist, 1975).

The *Escherichia coli* strain used to prepare pili was a pi-

† From the Division of Medical Genetics and Departments of Pathobiology and Medicine, University of Washington, and the Immunology Research Laboratory, U.S. Public Health Service Hospital, Seattle, Washington 98195. *Received August 2, 1977.* These investigations were supported in part by Public Health Service Grant R01-AI-13149 from the National Institute of Allergy and Infectious Diseases, by Federal Health Program Service Project SEA 76-43, and by Public Health Service grant GM-15253.

‡ Present address: Department of Biochemistry, Purdue University, West Lafayette, Indiana 47907.

0006-2960/78/0417-0442$01.00/0 

TABLE I: *Neisseria gonorrhoeae* Used to Prepare Pili, Strains F62, B, 33, and 7122.[a]

| Strain & pili type | COMP | MIFT | Auxotype |
|---|---|---|---|
| F62 | 2686 | B1 | $Pro^-$ |
| B | B | B2 | Zero |
| 33 | 33 | NT(c) | Zero |
| 7122 | 2686 | A | $A^-H^-U^-$ |

[a] COMP = serotyping by outer membrane protein–lipopolysaccharide complex. MIFT = serotyping by microimmunofluorescence test. Auxotype = typing of gonococci by nutritional requirements. $Pro^-$ = requires proline for growth. Zero = no defined nutritional requirements for growth. $A^-H^-U^-$ = requires arginine, hypoxanthine, and uracil for growth.

liated, nonflagellated strain (*E. coli* $B_{am}$ $p^+$) very generously supplied by Dr. Charles Brinton.

All organisms were grown on agar plates that contained G.C. medium base (Difco Laboratories, Detroit, Mich.) with 1% defined supplement (White & Kellogg, 1965) at 36.5 °C in an atmosphere of 5% $CO_2$.

*Purification of Gonococcal and Meningococcal Pili.* Gonococcal and meningococcal pili were purified utilizing the following method: Briefly, an 18–20-h growth of piliated organisms was harvested into ice-cold 10 mM Tris-HCl (Sigma Chemical Co., St. Louis, Mo.), pH 9.5, and sheared by vortexing (Vortex Genie Mixer, Scientific Products, McGaw Park, Ill.) for 5 min. First organisms and then large membrane fragments were removed by sequential centrifugations at 12 000*g* for 10 min and 50 000*g* for 1 h. The supernate of the 50 000*g* centrifugation was adjusted to 10% saturated ammonium sulfate (final) and left at 4 °C overnight. This suspension was then centrifuged at 30 000*g* for 10 min, and the pili pellet was carefully redissolved in water. Yields were 2–10 mg of pili/10 g of wet weight bacteria.

The *E. coli* pili were purified according to the method of Brinton (1965) using three cycles of precipitation in 0.1 M $MgCl_2$ and redissolving each pili precipitate by dialysis against 10 mM Tris-HCl buffer, pH 9.0, containing 0.02% $NaN_3$.

*Criteria for Purity of Pili.* Purity was assessed by sodium dodecyl sulfate–polyacrylamide disc or slab gel electrophoresis (Weber & Osborn, 1969). Proteins were identified by lightly sonicating the pili preparations for 1–3 min to expose tyrosine residues, radiolabeling with $^{125}I$ using a Chloramine-T procedure, and determining the radioactivity in the completed $NaDodSO_4$ gel electrophoresis[1] (Buchanan, 1975). Alternatively, some gels were stained with Coomassie Brilliant Blue (Buchanan, 1977). For all these sequence studies, only pili preparations with a pili protein radioactive peak $\geq 10 \times$ the highest other radioactive peak in the sample were utilized. Samples of high purity were dialyzed against distilled $H_2O$ to remove salts, lyophilized, and submitted for sequencing or compositional studies.

*Amino Acid Composition of Gonococcal Pili.* Pili from strain 33 were completely reduced and carboxamidomethylated as previously described (Chen et al., 1975). The modified pili were hydrolyzed in 6 N HCl at 108 °C for 24 and 96 h in evacuated tubes, and the hydrolyzates were dried in a vacuum desiccator over NaOH pellets. The analyses were performed on a Durrum D-500 automatic amino acid analyzer. Tryptophan was determined by the method of Liu and Chang (1971).

[1] Abbreviations used: Pth, phenylthiohydantoin; Dns, dansyl (5-dimethylaminonaphthalenesulfonyl); $NaDodSO_4$ gel electrophoresis, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

TABLE II: Amino Acid Composition of Pili 33.

| Amino acid | Residues/pilus subunit[a] |
|---|---|
| Asx | 17.9 |
| Thr[b] | 9.5 |
| Ser[c] | 13.7 |
| Glx | 19.7 |
| Pro | 5.8 |
| Gly | 13.2 |
| Ala | 17.7 |
| Val[d] | 16.3 |
| Met | 1.1 |
| Ile[d] | 8.4 |
| Leu[d] | 10.6 |
| Tyr | 5.6 |
| Phe | 1.7 |
| His | 2.1 |
| Lys | 14.6 |
| Arg | 6.9 |
| ½-Cystine[e] | 1.5 |
| Trp[f] | 2.6 |
| Total residues | 168.9 |

[a] The residue ratios were calculated for a molecular weight of 18 300. [b] Corrected for 5% loss during 24-h hydrolysis. [c] Corrected for 10% loss during 24-h hydrolysis. [d] The values of hydrophobic residues were obtained from HCl hydrolysis for 96 h. [e] The value of half-cystine was determined as carboxymethylcystine after complete reduction and carboxamidomethylation and not corrected for loss during 24-h hydrolysis. [f] Tryptophan was determined by the method of Liu & Chang (1971).

*Sequenator Methodology.* Pili protein (1–3 mg) was degraded in the Beckman 890C sequencer according to the methods of Hermodson et al. (1972) using a peptide program (Hermodson et al., 1977). The Pth-amino acids were identified by gas–liquid chromatography with the exceptions of the arginyl and histidyl derivatives which were identified by colorimetric spot tests (Hermodson et al., 1972). The stepwise yields for the degradations were 95–96%, and the yields of the Pth-amino acids in the early cycles of the run were more than 60% of those expected for the weight of protein degraded. The high pressure liquid chromatography was performed according to the method of Bridgen et al. (1976).

*Preparation of Dns-N-methylphenylalanine and Amino-Terminal Residue Determination.* The preparation of the dansyl derivative of *N*-methylphenylalanine (obtained as a generous gift from Drs. H. Neurath & K. A. Walsh, and subsequently also from Vega Fox Biochemicals, Tuscon, Ariz.) and the amino-terminal residue determination were performed according to Gray (1967). The dansyl amino acids were identified by thin-layer chromatography on polyamide sheets according to Woods & Wang (1967).

## Results

The amino acid composition of gonococcal pili protein (strain 33) is shown in Table II. The pili proteins from the other strains of gonococci had similar, but not identical, amino acid compositions.

When approximately 2 mg of gonococcal pili protein (strain F62) was degraded in the sequenator, two Pth-amino acids were observed in each cycle in approximately equimolar yield (50 nmol each, or roughly half the molar amount of protein). However, one of the pair was invariably present in the following cycle, and it was readily apparent that about half of the molecules lacked the amino-terminal amino acid present on the

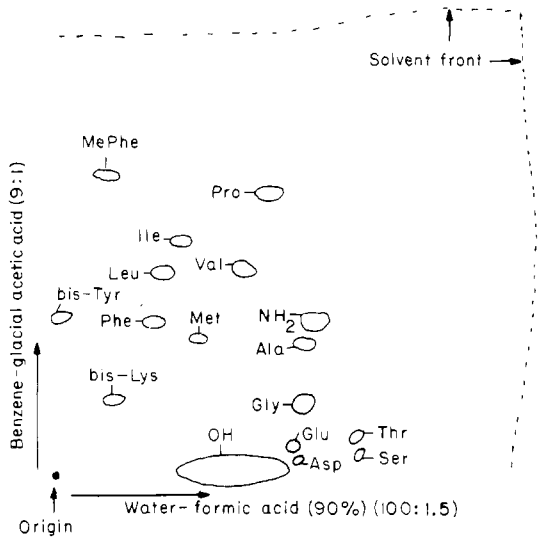


FIGURE 1: Separation of standard dansyl acids and dansyl-*N*-methylphenylalanine by two-dimensional thin-layer chromatography on polyamide sheets. The dansyl amino-terminus gonococcal pili, strain F62, cochromatographed with dansyl-*N*-methylphenylalanine (Dns-MePhe).

| | 1 | | | | 5 | | | | 10 | | | | 15 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| NEISSERIA [A]: | MePhe | Thr | Leu | Ile | Glu | Leu | Met | Ile | Val | Ile | Ala | Ile | Val | Gly | Ile |
| MORAXELLA [B]: | X | - | Thr | Leu | Ile | Glu | Leu | Met | Ile | Val | Ile | Ala | Ile | Ile | Gly | Ile |
| E. COLI [C]: | Ala | Ala | Thr | Thr | Val | Asn | Gly | Gly | Thr | Val | His | Phe | Lys | Gly | Glu | |

| | | | | | 20 | | | | 25 | | | | 29 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| NEISSERIA: | Leu | Ala | Ala | Val | Ala | Leu | Pro | Ala | Tyr | Gln | Asp | Tyr | Thr | Ala |
| MORAXELLA: | Leu | Ala | Ala | Ile | Ala | Leu | Pro | Ala | Tyr | Gln | Asp | Tyr | Ile | Ala |
| E. COLI: | Val | Val | Asn | Ala | Ala | ? | Ala | Val | Asp | - | - | - | - | - |

FIGURE 2: (A) Amino acid sequence of the pili protein of the four strains of *N. gonorrhoeae* and of strain 13090 of *N. meningitidis*. (B) Amino acid sequence of the pili protein of *Moraxella nonliquifaciens* from Frøholm & Sletten (1977). (C) Amino acid sequence of the pili protein of *E. coli*, strain $B_{amp}P^+$.

other half of the molecules. Since each amino acid except the first was present in two successive cycles, there was no difficulty in interpreting the data. This heterogeneity was present in the pili protein from all four strains of gonococci and also from the meningococci. There was no amino-terminal heterogeneity in the pili protein from *E. coli*.

The amino-terminal amino acid present on half the molecules was an unusual amino acid. When silylated by heating 10 min at 60 °C in 50% (v/v) *N,O*-bis(trimethylsilyl)acetamide in ethyl acetate, it gave two peaks in the gas chromatographic system used, one cochromatographing with Pth-glutamic acid and one chromatographing very near Pth-tyrosine. When chromatographed without silylation, all of the material appeared in the position where Pth-glutamic acid ran under silylated conditions, and the size of the peak corresponded to the sum of the two peaks observed under silylated conditions. This indicated that the earlier peak under silylated conditions was the unmodified Pth-amino acid, and the later peak (near Pth-tyrosine) was modified by the silylating agent. (Two peaks under silylating conditions are routinely observed in our system for Pth-glycine and Pth-proline, so this observation is not unique.)

High pressure liquid chromatography of this Pth-amino acid gave a peak appearing 1.0 min after Pth-lysine (the last Pth-amino acid normally seen in this system). The peak was collected, extracted into ethyl acetate, dried under a $N_2$ stream, and submitted for high resolution mass spectrographic analysis. The spectrogram had a mass ion at mass 296.0952 corresponding to a molecular formula of $C_{17}H_{16}ON_2S$, a fragment of mass 205.0418 corresponding to a molecular formula of $C_{10}H_9ON_2S$, fragments corresponding to benzyl and phenyl ions, and several smaller fragments. This analysis indicated that the compound was methylated Pth-phenylalanine, a finding entirely in agreement with the findings of Frøholm & Sletten (1977) of the unusual amino-terminal amino acid on the pili of *Moraxella nonliquifaciens*. These investigators had not completely determined the structure of the amino acid. The paucity of large fragments obtained in the mass spectrographic analysis made it impossible to unequivocally determine the structure of the unusual amino acid from that analysis alone. However, several observations had made it likely that the compound was the Pth derivative of *N*-methylphenylalanine. First, the mass spectrographic analysis was consistent with that structure (but not definitive). Second, the two-peak pattern of the silylated compound on the gas chromatograph (with the earliest peak being the unsilylated derivative) was entirely analogous to the situation for Pth-proline, the only ordinary secondary amino acid. Third, *N*-methylation of the amino terminus of the polypeptide was much more likely on biological grounds than incorporation of ring-methylated phenylalanine or benzylalanine into the protein, since *N*-methylation would very likely be a posttranslational step.

Thus, Pth-*N*-methylphenylalanine was prepared from D,L-*N*-methylphenylalanine. In both gas chromatographic systems (silylated and unsilylated) and in the high pressure liquid chromatograph, the synthetic Pth-*N*-methylphenylalanine comigrated with the Pth-amino acid isolated from the pili. In addition, the dansyl derivative cochromatographed with the dansyl-*N*-methylphenylalanine derived from the amino termini of the whole gonococcal pili (see Figure 1). The mass spectrogram of the synthetic Pth-*N*-methylphenylalanine also showed the same major fragments and mass ion as the material isolated from Edman degradation of the pili protein. Thus, we conclude that half the pili protein molecules begin with *N*-methylphenylalanine and half with threonine (the second amino acid in the longer protein).

The amino acid sequence from the meningococcal and four gonococcal pili proteins was very unusual (Figure 2). Only two of the first 24 residues were hydrophilic (residue 2:Thr, residue 5:Glu) and 20 of the 24 were aliphatic amino acids. The sequence was highly homologous to the *Moraxella nonliquifaciens* pili protein (Figure 2, Frøholm & Sletten, 1977). The pili protein from *E. coli* gave a totally unrelated sequence, however (Figure 2). No unusual features are apparent in this structure.

## Discussion

The amino-terminal amino acid sequence of the pili protein is highly conserved over strain and species differences while the antigenic determinants are not, implying that this region of the molecule is involved in an important way in the function of the protein. The highly hydrophobic character of the region makes it likely that this part of the polypeptide chain is located in one of two sites in the pilus structure: either buried deep inside the subunit or involved in subunit–subunit interactions. In either instance, maintenance of the hydrophobic character would be important to the structural integrity of the pilus, in

the former case in maintaining subunit structure and in the latter case in maintaining the polymeric structure.

Subtraction of the 29 amino terminal residues from the amino acid composition of the whole protein leaves a rather hydrophilic character for the rest of the molecule. That the antigenic determinants are in this hydrophilic portion is not surprising, since the antigenic determinants are presumably surface features.

Acknowledgments

The authors wish to thank Dr. William Trager of the Department of Pharmaceutical Chemistry, University of Washington, for assistance in the mass spectrographic analyses. We thank Drs. Hans Neurath and K. A. Walsh for providing access to the high pressure liquid chromatograph. Pamela Davick, Duane Olsen, and Linda Sires are thanked for technical assistance and Jeanne Arends is thanked for typing the manuscript.

References

Brinton, C. C. (1965) *Trans N.Y. Acad. Sci. 27,* 1003.

Brown, W. J., Lucas, C. T., & Kuhn, U. S. G. (1972) *Br. J. Vener. Dis. 48,* 177.

Buchanan, T. M. (1975) *J. Exp. Med. 141,* 1470.

Buchanan, T. M. (1977) in *The Gonococcus* (Roberts, R. B., Ed.) p 255, Wiley, New York, N.Y.

Buchanan, T. M., & Gotschlich, E. C. (1973) *J. Exp. Med. 137,* 196.

Buchanan, T. M., & Pearce, W. A. (1976) *Infect. Immun. 13,* 1483.

Buchanan, T. M., Swanson, J., & Gotschlich, E. C. (1972) *J. Clin. Invest. 51,* 17a.

Buchanan, T. M., Swanson, J., Holmes, K. K., Kraus, S. J., & Gotschlich, E. C. (1973) *J. Clin. Invest. 52,* 2896.

Bumgarner, L. C., & Finkelstein, R. A. (1973) *Infect. Immun. 8,* 919.

Carifo, K., & Catlin, B. W. (1973) *Appl. Microbiol. 26,* 223.

Catlin, B. W. (1973) *J. Infect. Dis. 128,* 178.

Chen, K. C. S., Kindt, T. J., & Krause, R. M. (1975) *J. Biol. Chem. 250,* 3280.

Devoe, I. W., & Gilchrist, J. E. (1975) *J. Exp. Med. 141,* 297.

Frøholm, L. D., & Sletten, K. (1977) *FEBS Lett. 73,* 29.

Gray, W. R. (1967) *Methods Enzymol. 11,* 13.

Hermodson, M. A., Ericsson, L. H., Titani, K., Neurath, H., & Walsh, K. A. (1972) *Biochemistry 11,* 4493.

Hermodson, M., Schmer, G., & Kurachi, K. (1977) *J. Biol. Chem. 252,* 6276.

James, A. N., Knox, J. M., & Williams, R. P. (1976) *Br. J. Vener. Dis. 52,* 129.

James-Holmquest, A. N., Swanson, J., Buchanan, T. M., Wende, R. D., & Williams, R. P. (1974) *Infect. Immunol. 9,* 897.

Jephcott, A. E., Reyn, A., & Birch-Andersen, A. (1971) *Acta Pathol. Microbiol. Scand., Sect. B, 79,* 437.

Johnston, K. H., Holmes, K. K., & Gotschlich, E. C. (1976) *J. Exp. Med. 143,* 741.

Kellogg, D. S., Peacock, W. L., Jr., Deacon, W. E., Brown, L., & Pirkle, C. I. (1963) *J. Bacteriol. 85,* 1274.

Kellogg, D. S., Cohen, I. R., Norins, L. C., Schroeter, A. L., & Reising, G. (1968) *J. Bacteriol. 96,* 596.

Knapp, J. S., & Holmes, K. K. (1975) *J. Infect. Dis. 132,* 204.

Koransky, J. R., Scales, R. W., & Kraus S. J. (1975) *Infect. Immun. 12,* 495.

Liu, T.-Y., & Chang, Y. H. (1971) *J. Biol. Chem. 246,* 2842.

Lucas, C. T., Chandler, F., Jr., Martin, J. E., Jr., & Schmale, J. D. (1971), *J. Am. Med. Assoc. 216,* 1612.

Mardh, P. A., & Westrom, L. (1976) *Infect. Immun. 13,* 661.

Novotny, P., & Turner, W. H. (1975) *J. Gen. Microbiol. 89,* 87.

Ofek, I., Mirelman, D., & Sharon, N. (1977) *Nature (London) 265,* 623.

Pearce, W. A., & Buchanan, T. M. (1978), *J. Clin. Invest.* (in press).

Punsalang, A. P., Jr., & Sawyer, W. D. (1973) *Infect. Immun. 8,* 255.

Schoolnik, G. K., Buchanan, T. M., & Holmes, K. K. (1976) *J. Clin. Invest. 58,* 1163.

Spackman, D. H., Stein, W. H., & Moore, S. (1958) *Anal. Chem. 30,* 1190.

Swanson, J. (1973) *J. Exp. Med. 137,* 571.

Swanson, J. S., Krause, S. J., & Gotschlich, E. C. (1971) *J. Exp. Med. 134,* 886.

Swanson, J., King, G., & Zeligs, B. (1975) *Infect. Immun. 11,* 453.

Waitkins, S. (1974) *Br. J. Vener. Dis. 50,* 272.

Wang, S. P., Holmes, K. K., Knapp, J. S., Ott, S., & Kyzer, D. D. (1977) *J. Immunol. 119,* 795.

Ward, M. E., Watt, P. H., & Robertson, J. N. (1974) *J. Infect. Dis. 129,* 650.

Weber, K., & Osborn, M. (1969) *J. Biol. Chem. 244,* 4406.

White, L. A., & Kellogg, D. S. (1965) *Appl. Microbiol. 13,* 171.

Wiesner, P. J., Handsfield, H. H., & Holmes, K. K. (1973) *N. Engl. J. Med. 288,* 1221.

Woods, K. R., & Wang, K. T. (1967) *Biochim. Biophys. Acta 133,* 369.