The Preparation and Properties of Gonococcal Pili

By JANET N. ROBERTSON, PHILIP VINCENT AND MICHAEL E. WARD

*Department of Microbiology, Southampton University Medical School,
Southampton General Hospital, Tremona Road, Southampton SO9 4XY*

(Received 28 March 1977)

Pili have been isolated from *Neisseria gonorrhoeae* by controlled homogenization followed by selective disaggregation in sucrose and purification by CsCl density gradient centrifugation. Pili from six gonococcal strains had buoyant densities of 1.30 to 1.31 g ml⁻¹ on CsCl. The pili were immunologically distinct when tested with rabbit antisera to purified pili. The amino acid composition of pilin from strains P9 and 201 was very similar, consisting of 208 and 212 amino acid residues respectively giving molecular weights of 22600 and 22352. The pili contained a high proportion (46 %) of non-polar amino acids. Further analysis of strain P9 pili revealed the presence of 1 to 2 phosphate groups and 1 to 2 hexose groups per pilin subunit; no amino sugars were detected. Pili from strain P9 were resolved into two bands by equilibrium density gradient centrifugation or column isoelectric focusing, suggesting the presence of more than one kind of pilus.

INTRODUCTION

Pili, protein filaments 7 nm in diameter, are universally present on the surface of gonococci on primary isolation from the patient. After subculture, non-piliated mutants soon outgrow the pilated gonococci indicating a selective pressure in favour of piliation in the natural infection. Laboratory studies suggest that pili may be important in several aspects of gonococcal virulence. Pilated gonococci show enhanced adhesion to the epithelium of the genital tract (Swanson, 1973; Ward & Watt, 1975) and may be able to migrate over the mucosal surface by the creeping motility demonstrated on agar surfaces (Henrichsen, 1975). Other reports suggest that pili protect gonococci against host defence systems, for example by increasing resistance to phagocytosis by polymorphs (Punsalang & Sawyer, 1973) and by reducing susceptibility to killing by antibody and complement (McCutchan, Levine & Braude, 1976). Thus pilus protein must be considered as a potential candidate for a vaccine against gonorrhoea. A further reason for studying the properties of pili is that they are immunogenic and may be a suitable antigen for the serological diagnosis of gonorrhoea (Buchanan *et al.*, 1973).

Gonococcal pili have been prepared by Buchanan and colleagues (Buchanan *et al.*, 1973; Buchanan, 1975) but little has been published on their properties. This paper describes the composition and properties of highly purified gonococcal pili prepared by an adaptation of methods devised for the purification of *Escherichia coli* sex pili (Beard, Howe & Richmond, 1972; Tomoeda, Inuzuka & Date, 1975).

METHODS

Bacterial strains. The strains of *Neisseria gonorrhoeae* used were selected because they were heavily pilated and relatively free from excessive budding of outer envelope material when examined by electron microscopy. Strains FA5 and 201 were kindly provided by Dr F. Sparling, Chapel Hill, North Carolina, U.S.A. Strains 17221, 24445, 24606 and P9 were obtained locally from patients attending a venereal disease clinic.

Cultures of *N. gonorrhoeae* were incubated overnight on Difco GC medium as previously described (Ward & Watt, 1971). Colonies of the pilated type 1 and 2 morphology were selectively subcultured (Kellogg *et al.*, 1963) and frozen in liquid nitrogen (Ward & Watt, 1971). For large-scale growth, gonococci direct from liquid nitrogen storage were inoculated on to 27×37 cm trays of Difco GC medium and incubated overnight in a humid atmosphere containing 5 % (v/v) CO₂.

Pilus purification. The gonococci were harvested from solid media into ice-cold 0.01 M-Tris/HCl buffer pH 8.0. To shear off the pili, the suspended gonococci were homogenized for 2 min in an Ultra Turrax homogenizer (Vortex Mixers, Thames St, Hampton, Middlesex) with a blade clearance of 0.025 mm. Optimum depilation conditions for this system were established by electron microscopy of negatively stained gonococci which had been homogenized for different times and then fixed in glutaraldehyde to prevent pilus regrowth. The sheared pili were disaggregated by adding sucrose at a final concentration of 1 M to the gonococcal suspension. The mixture was left on ice for 30 min and then homogenized for 15 s before centrifuging twice at 10000 g for 30 min. This ensured complete removal of intact bacteria without significant loss of pili. Outer envelope aggregates were removed from the mixture by centrifuging twice at 15000 g for 30 min. NaCl was then added to the sucrose-containing supernatant to a final concentration of 1 M and the impure pili were sedimented by centrifuging at 100000 g for 4 h at 4 °C. The pellet of impure pili was suspended in 0.01 M-Tris/HCl buffer pH 8.0 and CsCl was added to 32.5 % (w/w) (Beard *et al.*, 1972). The pili were dispersed by brief homogenization with a motor-driven Teflon pestle, centrifuged at 72000 g for 48 to 72 h at 5 °C in a swing-out rotor and the sharp pilus band at a buoyant density of 1.30 to 1.31 g ml⁻¹ was recovered. Any samples of pili found at this stage to be impure by dark-ground microscopy were subjected to a second CsCl density gradient centrifugation. Finally, the concentrated, purified pili were washed twice by suspension in 0.01 M-Tris/HCl buffer pH 7.2 containing 0.38 M-NaCl, and centrifuged at 100000 g for 1 h at 4 °C. The yield of purified pili varied considerably with the strain of gonococcus but was of the order of 1 mg of pilus protein per 10 g wet wt of gonococci.

Microscopy. Samples of pili or gonococci for electron microscopy were suspended in 0.01 M-Tris/HCl buffer pH 8.0 and negatively stained on Formvar-carbon grids by the technique of Kelen, Hathaway & McLeod (1971).

Dark-ground light microscopy of pili aggregated by salt or antibody was performed with an Ortholux II microscope (E. Leitz, Wetzlar, West Germany) equipped with an oil immersion dark-ground condenser.

Protein and phosphate estimations. Protein was estimated by the method of Lowry *et al.* (1951) and phosphate by the method of Chen, Toribara & Warner (1956).

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis. Pilus preparations were dissociated for 2 h at 37 °C with 2 % (w/v) buffered sodium dodecyl sulphate (SDS). Samples were electrophoresed in glass tubes as described by Heckels (1977), using a 3 % (w/v) polyacrylamide stacking gel and a 10 or 12 % running gel. Molecular weights of the pilus subunits on SDS-polyacrylamide gel electrophoresis were calculated by the method of Weber & Osborn (1969) using egg lysozyme, ribonuclease, myoglobin, trypsin soy bean inhibitor, bovine serum albumin, β -lactoglobulin, ovalbumin and trypsin as molecular weight standards.

Micro-isoelectric focusing. A sample (1 mg) of purified pili was dialysed against 1 % (w/v) glycine in distilled water (Beard *et al.*, 1972) and mixed with the heavy solution of a sucrose gradient. The sample was electro-focused as described by Kint (1975) using a column of 20 ml capacity cooled to 1 °C, a sucrose gradient of 5 to 50 % (w/v) sucrose and 3 % (v/v) of an equal mixture of ampholines pH 3.5 to 6.0 and pH 5.0 to 8.0 (LKB). A potential of 400 to 800 V was applied over 16 to 22 h until the current was ≤ 1 mA and the sample bands were stationary. In some experiments control proteins (human and sheep haemoglobin) of known pI were added to the samples.

Amino acid and amino sugar analysis. Samples (10 nmol) of purified pili in nitrogen-flushed tubes were hydrolysed in 6 M-HCl under vacuum for 16 to 32 h at 105 °C. Tryptophan was determined in the presence of 4 % (v/v) thioglycolic acid by the method of Matsubara & Sasaki (1969). Analyses were carried out on a JEOL 6AH automatic amino acid analyser (Japanese Electron Optics, Tokyo, Japan). The conditions of hydrolysis were checked and the amino acid recoveries were corrected by processing a sample of purified egg lysozyme in parallel. At least four batches of pili from each strain were analysed and good agreement was obtained.

Samples of purified p9 pili (400 μ g) and of a control protein known to contain amino sugar (ovalbumin) were hydrolysed in 4 M-HCl for 4 h under nitrogen at 105 °C to release amino sugar. The pilus and control hydrolysates were then analysed for amino sugars using a JEOL 5AH amino acid analyser.

Neutral sugar analysis. Samples (450 μ g) of purified p9 pili or of ovalbumin (control) were hydrolysed in 2 M-HCl for 2 h under nitrogen at 105 °C to destroy glycosidic bonds and release the sugars. The hydrolysate was neutralized with Amberlite IR 45 (OH⁻ form, 40 to 60 mesh) ion exchange resin. The pH of the solution was controlled and resin was added to maintain pH 4 to 5. The liquid plus washings were then chromatographed on a 30×8 mm column of Amberlite CG 120 (H⁺ form, 200 to 400 mesh) and the eluate, from which amino acids had now been removed, was collected and freeze-dried. The weight of neutral sugars detected in

Fig. 1. Electron micrograph of purified gonococcal pili negatively stained with uranyl acetate. Note the absence of any contaminating vesicular material characteristic of gonococcal cytoplasmic membrane or outer envelope components. Bar marker represents 200 nm.

the column eluate by the phenol-sulphuric acid reaction (Dubois *et al.*, 1956) was 5.2 μg corresponding to 1.3 % (w/w) of the pili hydrolysed. A further sample of the eluate was silylated with trisilyl imidazole (Pierce Chemical Co., Rockford, Illinois, U.S.A.) and analysed on a Pye Unicam 104 gas liquid chromatograph. The samples were chromatographed on a 4×2.1 mm glass column of 3 % (w/w) OV1 on Diatomite CQ (Pye Unicam) with nitrogen as carrier gas at 45 ml min^{-1} and at a programmed temperature of 100 $^{\circ}\text{C}$, increasing at 2 $^{\circ}\text{C min}^{-1}$ to 230 $^{\circ}\text{C}$.

Antisera. Male New Zealand White rabbits (about 3 kg) were injected subcutaneously with 50 μg of purified p9 or 201 pili in Freund's incomplete adjuvant (Difco) at 0, 2, 4, 6 and 8 weeks. High levels of anti-pilus antibodies were present in the sera 10 weeks after commencing immunization.

Pilus agglutination. Samples (3 μl) of a pilus preparation containing 500 μg protein ml^{-1} in 0.01 M-Tris/HCl buffer pH 8.0 were mixed on a microscope slide with 3 μl of buffer (control) or an antiserum dilution in buffer. After incubation for 1 h at 37 $^{\circ}\text{C}$ in a moist chamber the preparations were examined for pilus agglutination by dark-ground light microscopy (Brinton, 1975).

RESULTS

Pili stick readily to the hydrophobic surface of Formvar-carbon electron microscope grids whereas contaminating gonococcal cell debris does not. Thus conventional methods of negative staining for electron microscopy may give a false impression of purity. This problem was overcome by using the agar slide technique of Kelen *et al.* (1971) which concentrates and traps, by gel filtration, the macromolecules and larger debris present in the sample. A typical electron micrograph of purified p9 pili is shown in Fig. 1. Pili were not accepted as pure until free from detectable debris using this technique.

Gonococcal pili have a diameter of about 7 nm; thus individual pili found in environments of low ionic strength are invisible under the light microscope. However, at high ionic strength the pili aggregate into needle-like crystals of parallel filaments (Fig. 2) which, like *E. coli* sex pili (Brinton, 1971), can be resolved by dark-ground light microscopy.

Fig. 2. Crystals of highly purified, salt-aggregated gonococcal pili resolved by dark-ground light microscopy. 'Rafts' of aggregated vesicles of contaminating outer envelope components are absent from the preparation. The addition of 1 M-sucrose to such a preparation of pilus crystals disaggregates the pilus bundles to individual pili which can no longer be visualized by light microscopy. Bar marker represents 20 μm .

Contaminating wall debris also aggregates in the presence of high salt concentrations giving rise to visible clumps of vesicular material. The absence of such material from preparations of salt-aggregated pili examined by dark-ground microscopy (Fig. 2), although only qualitative, is a convenient and sensitive criterion of purity.

Centrifugation of pili on a continuous CsCl density gradient resulted in sharp pilus bands with buoyant densities of 1.30 to 1.31 g ml^{-1} according to the strain used. In many strains, including P9, the pili formed two distinct, closely adjacent bands indicating the presence of two different populations of pili (Fig. 3). After re-mixing, the same P9 pili were analysed by isoelectric focusing. Two distinct bands of pili were again formed with isoelectric points of pI 4.9 and 5.3 (Fig. 4).

After SDS-polyacrylamide gel electrophoresis, purified pili consistently gave a single band which, on staining with Coomassie blue, corresponded to the gonococcal pilin subunit. Dissociation of pili to the subunit by SDS/mercaptoethanol treatment at either 37 °C for 2 h or 100 °C for 10 min did not alter the pilin subunit molecular weight. However, slightly different molecular weights were obtained depending on whether a 10 % or 12 % polyacrylamide running gel was used, a phenomenon noted with other proteins (Dunker & Rueckert, 1969). Pilin from six strains of gonococci all had similar molecular weights in the range 18000 to 20000 when analysed by SDS-polyacrylamide gel electrophoresis (Table 1).

Table 2 shows the amino acid composition of purified P9 and 201 pili. In both strains the pilin subunit had approximately the same number of amino acid residues, 208 in strain P9 and 212 in strain 201, giving calculated molecular weights of 22600 for P9 and 22352 for 201. The molar ratios of the amino acids for the two strains were also virtually identical with the exception of proline. No amino sugars were detected in a hydrolysate of purified P9 pili using a specially programmed amino acid analyser even though in a parallel experiment 83 % of the glucosamine in an ovalbumin control was detected. Samples (450 μg) of P9 pili were hydrolysed to destroy any glycosidic bonds present and the amino

Fig. 3. Separation and purification of crude gonococcal pili by CsCl density gradient ultracentrifugation. The pili were evenly suspended in 32.5 % (w/w) CsCl in 0.01 M-Tris/HCl buffer pH 8.0 and centrifuged in a swing-out rotor at 72000 g for 48 h. Two closely adjacent bands of highly purified pili were consistently obtained from strain p9 gonococci and from other gonococcal strains.

Fig. 4. Separation of purified strain p9 gonococcal pili by small-scale analytical isoelectric focusing in a specially constructed column. A preparation of purified pili was extensively dialysed against 1 % (w/v) glycine and electrofocused at 1 °C in a mixture of ampholytes from pH 3.5 to 8.0, in a sucrose gradient of 5 to 50 % (w/v) sucrose, at a potential difference of 400 to 800 V for 22 h. Two different bands of pili with isoelectric points of pI 4.9 and pI 5.3 were obtained suggesting that a single gonococcal strain may carry more than one kind of pilus.

acids were removed from the hydrolysate by ion exchange chromatography. The weight of remaining neutral sugars, determined by the phenol-sulphuric acid reaction (Dubois *et al.*, 1956), was 5.2 μ g, i.e. 1.3 % (w/w) of pili hydrolysed. Qualitative gas chromatographic analysis of a portion of this carbohydrate revealed the presence of galactose and also traces of glucose. No heptose or pentose was detected.

Phosphate analysis of purified p9 pili by the method of (Chen *et al.*, 1956) revealed the presence of 1 to 2 phosphate groups (actual result 1.7) per pilin subunit.

Table 1. *Molecular weights determined by SDS-polyacrylamide gel* electrophoresis of pilin from six strains of N. gonorrhoeae*

Strain	Mol. wt.
P9	19 500
FA5	19 500
17221	18 120
24445	18 580
24606	18 700
201	19 600

* On 12 % (w/v) polyacrylamide.

Table 2. *Amino acid composition of purified gonococcal strains P9 and 201 pili*

Results indicate the number of amino acid residues per pilin subunit. Other components in P9 pili were 1 to 2 residues of phosphate and 1 to 2 residues of hexose per pilin subunit.

Residue	Strain		Residue	Strain	
	P9	201		P9	201
Asx	31	30	Arg	8	4
Ala	24	27	Ile	6	6
Glx	21	20	Trp	6	4
Val	18	18	Tyr	5	6
Gly	17	18	Cys/2	5	4
Ser	16	18	Met	4	2
Lys	16	16	His	3	3
Thr	12	14	Phe	3	2
Leu	12	13	Pro	1	7
			Total	208	212
			Mol. wt	22 600	22 352

DISCUSSION

Buchanan and colleagues (Buchanan *et al.*, 1973; Buchanan, 1975) were the first to prepare useful quantities of gonococcal pili by sedimentation near their isoelectric point followed by precipitation with 0.1 M-Mg²⁺ or 10 % (v/v) saturated ammonium sulphate solution. The major problem with this technique is that gonococci produce abundant outer envelope material in the form of vesicles which co-precipitate with the pili and become enmeshed in the pilus bundles. This problem has been overcome by Brinton (see Beard *et al.*, 1972) for *E. coli* sex pili by using 30 % sucrose to disperse the pilus bundles. The lectin-like, pilus-mediated haemagglutination of red blood cells by *Salmonella typhimurium* and *Shigella flexneri* type 1 pili is also inhibited by a carbohydrate, D-mannopyranose (Old, 1972). Thus it might be that the mechanism by which pili aggregate to each other is similar to the way in which they interact with host cell surfaces to facilitate attachment.

Brinton (1971) has suggested that the phosphate present in the *E. coli* F pilus subunit may provide energy for the polymerization of pilin subunits to form the pilus strand, or to orientate the subunits correctly for pilus synthesis. Phosphate analysis of our preparations of P9 pili, free from detectable contamination with nucleic acids, revealed similar amounts of phosphate of the order of 1 to 2 phosphate groups per pilin subunit. This phosphate would alter the overall charge and increase the electrophoretic mobility of the pilin subunit, thus explaining why the molecular weights estimated from SDS-polyacrylamide gel electrophoresis were lower than those calculated from the amino acid analyses.

Escherichia coli sex pili are glycoproteins; Brinton (1971) reported the presence of one molecule of D-glucose in the F pilin subunit whilst Beard, cited by Tomoeda *et al.* (1975),

Table 3. Comparison of the amino acid characters of *N. gonorrhoeae* strains P9 and 201 pili with *E. coli* common (type 1) and sex (F) pili (Brinton, 1965, 1971)

Amino acid character	Percentage of character in pilin from:			
	<i>N. gonorrhoeae</i>		<i>E. coli</i>	
	P9	201	F	type 1
Basic*	13	10.8	8.1	4.9
Acidic†	25	23.6	9.7	20.2
Total non-polar‡	45.7	45.3	66.9	54
Essentially hydrophobic§	26	24	42.7	22.7

* Lys, Arg, His.

† Glu, Asp.

‡ Val, Leu, Met, Phe, Ile, Tyr, Trp, Gly, Ala.

§ Val, Leu, Met, Phe, Ile, Tyr, Trp.

has identified one molecule each of D-glucose, glucosamine and galactose in the R1drd19 sex pilin subunit. Comparable amounts (1.3 %, w/w) of carbohydrate were detected in purified gonococcal pili. Qualitative gas chromatographic analysis showed that galactose and traces of glucose were present but there was no detectable heptose or amino sugars. This suggests that the preparations were not contaminated with gonococcal lipopolysaccharide whose carbohydrate contains, besides glucose, and galactose, glucosamine and heptose (Perry *et al.*, 1975). Current evidence indicates that there is a large but undetermined number of serotypes of gonococcal pili (Novotny & Turner, 1975). The role of the sugar group in the antigenicity of the pilus remains to be determined.

Gonococci must adhere to the mucosal surfaces of the host in order to resist the shearing forces which result from urine and mucus flows. This attachment was confirmed by electron micrographs of exfoliated cells which revealed large numbers of gonococci adherent to epithelial and mucous secreting cells (Ward & Watt, 1972). There is now abundant evidence that pili facilitate the attachment of gonococci to the mucosal surfaces of the genital tract (Ward, Watt & Robertson, 1974; Mårdh & Weström, 1976) but this may be by a non-specific mechanism (Koransky, Scales & Kraus, 1975). The relative proportions of hydrophilic and hydrophobic amino acids of strains P9 and 201 gonococcal pili and those of *E. coli* common (type 1) and sex (F) pili (Brinton 1965, 1971) are similar (Table 3). (In this table all the Asx and Glx residues have been assumed to represent the acidic amino acids aspartate and glutamate. Tyrosine and tryptophan have been included as hydrophobic amino acids even though they also have the capacity to interact favourably with water). The high proportion of hydrophobic amino acids can explain the ability of pili to bind to each other and suggests that they could attach to mucosal surfaces by anchoring in the hydrophobic interior of the host cell membrane. Indeed, Kihlström & Edebo (1976) have demonstrated that surface hydrophobicity is an important determinant in the attachment of *Salmonella typhimurium* to HeLa cells.

Work in this laboratory has shown that long range electrostatic repulsion between the negatively charged gonococcal and host cell surfaces is normally an important barrier to gonococcal attachment; pili only facilitate attachment when long range electrostatic repulsion is significant (Heckels *et al.*, 1976). Purified P9 pili have an isoelectric point identical to whole pilated P9 gonococcal cells (pI 5.3) and slightly less than non-pilated P9 gonococci (pI 5.6). Thus pili may dominate the surface charge of the gonococcus and in themselves will not be more electrostatically attractive to the host cell surface than the rest of the gonococcal surface. However, the presence of large amounts of non-polar amino acids in the pili (Table 3) may mean that their charge density is less than that of other gonococcal surface components. This, together with their small surface area, would reduce the electro-

static repulsion of the pili compared with the whole gonococcal surface, thus facilitating attachment.

Pilus-mediated attachment to the human fallopian tube in organ culture (Watt & Ward, 1977) and to red cells (Buchanan & Pearce, 1976) is decreased by specific antibodies to the pili. Thus, if pili are a critical determinant of gonococcal attachment to mucosal surfaces in the natural infection, blocking this adhesion with antibodies would be the ideal form of immunization as the carrier state would also be prevented. The promise of this approach is shown by the reports of Brinton (1975) that the infectious dose of laboratory-grown gonococci for volunteers is raised several fold after immunization with pure pili. A serious difficulty in the preparation of a pilus vaccine is the demonstration of considerable serological heterogeneity among pili (Novotny & Turner, 1975). Buchanan & Pearce (1976) also demonstrated antigenic heterogeneity in the four gonococcal pilus preparations tested; in a radio-immunoassay the degree of shared antigenicity amongst these preparations was estimated to be $\leq 2.5\%$ (w/w). This serological heterogeneity of gonococcal pili was confirmed in our own experiments; rabbit antisera to purified P9 or 201 pili with agglutination titres for the homologous pili of 1:64000 and 1:18000 respectively, failed at a dilution as low as 1:20 to agglutinate purified pili from four heterologous gonococcal strains. The minor differences observed in the amino acid composition of strain P9 and 201 gonococcal pili might be sufficient to explain why they are serologically distinct yet still retain a common function. However, the overall similarity in their amino acid composition suggests that on peptide mapping and sequencing, it will be found that gonococcal pilins from different strains have a large homologous region of constant sequence and a small area of variable sequence determined by the pilus serotype. One possibility is that human beings, unlike rabbits, respond to a common antigenic determinant on gonococcal pili. This would explain why antibodies to a single strain of pili were detected in the sera from 90% of women with gonorrhoea (Buchanan *et al.*, 1973). Further work is needed to establish this point since there remains the alternative possibility that the pilus preparations used in published serological tests were contaminated with serologically significant amounts of cross-reacting antigens.

Clearly, pili hold some promise as a solution to the problem of developing a gonococcal vaccine. Future research will doubtless be directed towards identifying the component on the pilin subunit responsible for adhesion to host cell membranes and to establishing if this is immunogenic for man.

We are grateful to Professor P. J. Watt for his interest and encouragement and to the Medical Research Council for a programme grant. We thank the Department of Physiology and Biochemistry, Southampton University, for making available their facilities for amino acid analyses.

REFERENCES

- BEARD, J. P., HOWE, T. G. B. & RICHMOND, M. H. (1972). Purification of sex pili from *E. coli*. *Journal of Bacteriology* **111**, 814-820.
- BRINTON, C. C. (1965). The structure, function, synthesis and genetic control of bacterial pili and a molecular model for DNA and RNA transport in gram negative bacteria. *Transactions of the New York Academy of Sciences* **27**, 1003-1054.
- BRINTON, C. C. (1971). The properties of sex pili, the viral nature of 'conjugal' genetic transfer systems, and some possible approaches to the control of bacterial drug resistance. *CRC Critical Reviews in Microbiology* **1**, 105-160.
- BRINTON, C. C. (1975). Current direction in gonococcal research. Annual Meeting of American Society for Microbiology, Seminar, April 28, New York.
- BUCHANAN, T. M. (1975). Antigenic heterogeneity of gonococcal pili. *Journal of Experimental Medicine* **141**, 1470-1475.
- BUCHANAN, T. M. & PEARCE, W. A. (1976). Pili as a mediator of the attachment of gonococci to human erythrocytes. *Infection and Immunity* **13**, 1483-1489.

- BUCHANAN, T. M., SWANSON, J., HOLMES, K. K., KRAUS, S. J. & GOTSCHLICH, E. C. (1973). Quantitative determination of antibody to gonococcal pili. Changes in antibody levels with gonococcal infection. *Journal of Clinical Investigation* **52**, 2896-2909.
- CHEN, P. S., JR, TORIBARA, T. Y. & WARNER, H. (1956). Micro determination of phosphorus. *Analytical Chemistry* **28**, 1756-1758.
- DUBOIS, M., GILLES, K. A., HAMILTON, J. K., REBERS, P. A. & SMITH, F. (1956). Colorimetric method for the determination of sugars and related substances. *Analytical Chemistry* **28**, 350-356.
- DUNKER, A. K. & RUECKERT, R. R. (1969). Observations on molecular weight determinations on polyacrylamide gel. *Journal of Biological Chemistry* **244**, 5074-5080.
- HECKELS, J. E. (1977). The surface properties of *Neisseria gonorrhoeae*: isolation of the major components of the outer membrane. *Journal of General Microbiology* **99**, 333-341.
- HECKELS, J. E., BLACKETT, B., EVERSON, J. S. & WARD, M. E. (1976). The influence of surface charge on the attachment of *Neisseria gonorrhoeae* to human cells. *Journal of General Microbiology* **96**, 359-364.
- HENRICHSEN, J. (1975). The occurrence of twitching motility among gram-negative bacteria. *Acta pathologica et microbiologica scandinavica* **B83**, 171-178.
- KELN, A. E., HATHAWAY, A. E. & MCLEOD, D. A. (1971). Rapid detection of Australia/SH antigen and antibody by a simple and sensitive technique of immuno electron microscopy. *Canadian Journal of Microbiology* **17**, 993-1000.
- KELLOGG, D. S., PEACOCK, W. L., DEACON, W. E., BROWN, L. & PIRKLE, C. I. (1963). *Neisseria gonorrhoeae*. I. Virulence genetically linked to clonal variation. *Journal of Bacteriology* **85**, 1274-1279.
- KIHLSTRÖM, E. & EDEBO, L. (1976). Association of viable and inactivated *Salmonella typhimurium* 395 MS and MR 10 with HeLa cells. *Infection and Immunity* **14**, 851-857.
- KINT, J. A. (1975). A simple microtechnique for isoelectric focusing on a density gradient. *Analytical Biochemistry* **67**, 679-683.
- KORANSKY, J. R., SCALES, R. W. & KRAUS, S. J. (1975). Bacterial haemagglutination by *Neisseria gonorrhoeae*. *Infection and Immunity* **12**, 495-498.
- LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L. & RANDALL, R. J. (1951). Protein measurement with the Folin phenol reagent. *Journal of Biological Chemistry* **193**, 265-275.
- MÄRDH, P. A. & WESTRÖM, L. (1976). Adherence of bacteria to vaginal epithelial cells. *Infection and Immunity* **13**, 661-666.
- MATSUBARA, H. & SASAKI, R. M. (1969). High recovery of tryptophan from acid hydrolysates of proteins. *Biochemical and Biophysical Research Communications* **35**, 175-181.
- MCCUTCHAN, J. A., LEVINE, S. & BRAUDE, A. I. (1976). Influence of colony type on susceptibility of gonococci to killing by human serum. *Journal of Immunology* **116**, 1652-1655.
- NOVOTNY, P. & TURNER, W. H. (1975). Immunological heterogeneity of pili of *Neisseria gonorrhoeae*. *Journal of General Microbiology* **89**, 87-92.
- OLD, D. C. (1972). Inhibition of the interaction between fimbrial haemagglutinins and erythrocytes by D-mannose and other carbohydrates. *Journal of General Microbiology* **71**, 149-157.
- PERRY, M. B., DAOUST, V., DIENA, B. B., ASHTON, F. E. & WALLACE, R. (1975). The lipopolysaccharides of *Neisseria gonorrhoeae* colony types 1 and 4. *Canadian Journal of Biochemistry* **53**, 623-629.
- PUNSALANG, A. P. & SAWYER, W. D. (1973). Role of pili in the virulence of *Neisseria gonorrhoeae*. *Infection and Immunity* **8**, 255-263.
- SWANSON, J. (1973). Studies on gonococcus infection. IV. Pili: their role in attachment of gonococci to tissue culture cells. *Journal of Experimental Medicine* **137**, 571-589.
- TOMOEDA, M., INUZUKA, M. & DATE, T. (1975). Bacterial sex pili. *Progress in Biophysics and Molecular Biology* **30**, 23-56.
- WARD, M. E. & WATT, P. J. (1971). The preservation of gonococci in liquid nitrogen. *Journal of Clinical Pathology* **24**, 122-123.
- WARD, M. E. & WATT, P. J. (1972). Adherence of *Neisseria gonorrhoeae* to urethral mucosal cells: an electron microscopic study of human gonorrhoea. *Journal of Infectious Diseases* **126**, 601-605.
- WARD, M. E., WATT, P. J. & ROBERSTON, J. N. (1974). The human fallopian tube: a laboratory model for gonococcal infection. *Journal of Infectious Diseases* **129**, 650-659.
- WARD, M. E. & WATT, P. J. (1975). Studies on the cell biology of gonorrhoea. In *Genital Infections and their Complications*. Edited by D. Danielsson, L. Juhlin and P. A. Mårdh. Stockholm: Almqvist & Wiksell.
- WATT, P. J. & WARD, M. E. (1977). The interaction of gonococci with human epithelial cells. In *The Gonococcus*. Edited by R. Roberts. New York: Wiley (in the Press).
- WEBER, K. & OSBORN, M. (1969). The reliability of molecular weight determinations by dodecyl sulphate-polyacrylamide gel electrophoresis. *Journal of Biological Chemistry* **244**, 4406-4412.