CHAPTER V

CONCLUSION

The present investigations attempt to examine the purified gonococcal pili antigen in clinical isolated Neisseria. Thirteen piliated gonococci were selected from thirty-one clinical isolates on the basis of colony typing system and electron microscopy. The former method remains the useful test for sorting out piliated gonococci from gonococcal population. Purification of pili was encountered with protein contamination,poor output and time consumption. Only five pili preparations out of twelve piliated strains could be obtained, at a low yield of 0.22 to 1.03 mg/10 g wet weight of bacteria.

Visualization of gonococcal pili under electron microscopy revealed long filamentous strands with a diameter of 7 nm.The 5 pili preparations retain their morphology after purification process and are indistinguishable both among themselves and from E. coli pili. Most pili preparations showed a single band upon SDS-Polyacrylamide Gel Electrophor esis from which the subunit molecular weights were calculated (ranging from 18,000 to 22,500 daltons).

Antigenicity and immunogenicity of pilin were

explored by raising anti-pili antibody for ELISA test. Immunization of one pili preparation in rabbits could elicit a good antibody response with a titer of 1:81920 by Indirect Hemagglutination. This antibody afforded high specificity when tested against other bacteria by Coagglutination and was further used for the detection of pili antigen by ELISA. Only one pili out of 4 heterologous pili preparation and 4 out of 35 piliated gonococci were reactive with this anti-pili antibody by ELISA system, indicating extreme pili heterogeneity among strains. It is also indicative of high specificity of rabbit antibody against a variable epitope on the immunized pilin.

The high specificity of antipili antibody and pili heterogeneity presents problems in developing an ELISA system for pili antigen determination. Therefore, the use of rabbit antipili against a single local strain of gonococcal pili could not be feasible for the diagnosis of gonococcal infection. The employing of polyvalent anti-pili antibodies from pools of antisera against different strains and/or immunization of purified common fragment of gonococcal pili may be solutions to increase the capacity for pili antigen detection. Nevertheless, antipili-antibodies could be invaluable in the analysis of gonococci for taxonomic and epidemiological purposes.

Table 1 Molecular Weights of Pilin as Determined by SDS-Polyacrylamide Gel Eletrophoresis (113).

N.gonorrhoeae strain	Molecular Weight
MS 11 (Tr)	17,500
MS 11 (Op)	17,500
R 10 (Tr)	16,400
R 10 (Op)	17,000
R 16 (Tr)	16,200
R 16 (Op)	17,500
2686 (Tr)	18,000
2686 (Op)	18,000

Tr = Transparent Op = Opaque

Table 2 Amino-Terminal Amino Acid Sequences of Pili Protein from Isogenic Transparent (Tr) clones of Gonococcal Strains MS 11 and R10 and from Moraxella nonliquefaciens, Pseudomonas aeruginosa and Escherichia coli (113).

	1				5					10					15					20
N.gonorrhoeae	MePhe	Thr	Leu	Ile	Glu	Leu	Met	Ile	Val	Ile	Ala	Ile	Val	Gly	Ile	Leu	Ala	Ala	Val	Ala
N.nonliquefaciens	MePhe	Thr	Leu	Ile	Glu	Leu	Met	Ile	Val	Ile	Ala	Ile	Ile	Gly	Ile	Leu	Ala	Ala	Val	Ala
P.aeruginosa	MePhe	Thr	Leu	Ile	Glu	Leu	Met	Ile	Val	Val	Ala	Ile	Ile	Gly	Ile	Leu	Ala	Ala	Val	Ala
E.coli	Ala	Ala	Thr	Thr	Val	Asn	Gly	Gly	Thr	Val	His	Phe	Lys	Gly	Glu	Val	Val	Asn	Ala	Ala
					25					30					35					40
N.gonorrhoeae	Leu	Pro	Ala	Tyr	Gln	Asp	Tyr	Thr	Ala	Arg	Ala	Gln	Val	Ser	Glu	Ala	Ile	Leu	Leu	Ala
N.nonliquefaciens	Leu	Pro	Ala	Tyr	Gln	Asp	Tyr	Ile	Ala	Arg	Ala	Gln	Val	Ser	Glu	Ala	Phe	Thr	Leu	Ala
P.aeruginosa	Ile	Pro																		
E.coli	-?-	Ala	Val	Asp																
					45					50					55					59
N.gonorrhoeae	Glu	Gly	Gln	Lys	Ser	Ala	Val	Thr	Glu	Tyr	Tyr	Leu	Asn	His	Gly	Lys	Trp	Pro	Glu	
N.nonliquefaciens	Asp	Gly	Leu	Lys	Thr	Gly	Ile	Ser	Thr											

Table 3 Characteristics of Colony Types of N.gonorrhoeae(55,123).

Type	Size (mm)	Elevation	Color density	Edges	Opacity	Structure	Consistency
1	0.5	convex	dark gold	entire	translucent	amorphous	slightly viscid
2	0.5	convex	dark gold	defined crenated	translucent	amorphous	friable
3	1.0	low convex	light brown	entire	translucent	granular	viscid
4	1.0	low convex	colorless	entire	transparent	amorphous	viscid
5	1.0	low	dark brown	coarsely	opaque	granular	-

Table 4 Composition of SDS-Polyacrylamide Gels.

Solutions (ml)	%Acrylamide	
	Stacking gel 5%	Separating gel 12.5%
Stock acrylamide	0.835	4.167
1.5M Tris-HCl pH 8.8	-	2.500
0.5M Tris-HCl pH 6.8	1.250	-
20% SDS	0.050	0.100
0.2M EDTA	0.050	0.100
Distilled water	2.710	2.925
TEMED	0.0025	0.005
10% $(NH_4)_2S_2O_8$	0.100	0.200
Total volume (ml)	5.000	10.000

Table 5 Relationship of Colony Type to Presence of Pili among Strains of N.gonorrhoeae.

Pili by EM	Colony type		Total (isolates)
	1 or 2	3,4,or5	
Positive	13	0	13
Negative	0	18	18
Total (isolates)	13	18	31

Chi-Square test at df=1, $P<0.01$

Table 6 Subunit Molecular Weights and Yields of Gonococcal Pili from Gonococcal Isolates.

Gonococcal isolates	Surface pili by EM	Pilin molecular weight	Wet weight of bacteria(g)	Pilin protein concentration (mg)	
				per 10 g wet weight	per 0.5 ml
K 210129	+	–	7.0,6.8	–	–
J 070229	+	18,000	7.3,7.1	0.30	0.22
S 280229	+	22,500 19,500	7.3,6.7	0.22	0.16
S 200329	+	18,750	8.0,7.1	0.51	0.31
P 010429	+	–	6.7,6.9	–	–
M 100429	+	–	7.3,7.1		
J 120529	+	–	8.0,7.3	–	–
S 040629	+	21,500	7.6,7.2,6.9 7.1,6.7	1.03	0.79
S 160829	+	18,000	4.5,5.2	0.31	0.19
S 210929	+	–	6.5,6.8	–	–
S 101229	+	–	8.0,7.4	–	–
S 230130	+	–	5.0,5.6	–	–

Table 7 Distribution of Optical Density Values of Bacterial Isolates in ELISA.

Antigen	No. of isolates yielding OD_{405}								No. positive for pili antigen
	0.000-0.049	0.050-0.099	0.100-0.149	0.150-0.199	0.200-0.249	0.250-0.299	0.300-0.349	0.350-0.400	
N.gonorrhoeae	0	15	74	8	2	2	1	0	5 (4.9%)
N.meningitidis	0	0	6	0	0	0	0	0	0
N.sicca	0	0	4	0	0	0	0	0	0
N.mucosa	0	0	3	0	0	0	0	0	0
B.catarrhalis	0	0	1	0	0	0	0	0	0
M.osloensis	0	0	1	0	0	0	0	0	0
P.aeruginosa	0	0	1	0	0	0	0	0	0
E.coli	0	0	1	0	0	0	0	0	0
S.aureus	0	0	1	0	0	0	0	0	0

Table 8 Pili Detection : Electron Microscopic Examination as Compared to ELISA.

ELISA	Pili by EM		Total (isolates)
	Positive	Negative	
Positive	4	1	5
Negative	31	66	97
Total (isolates)	35	67	102

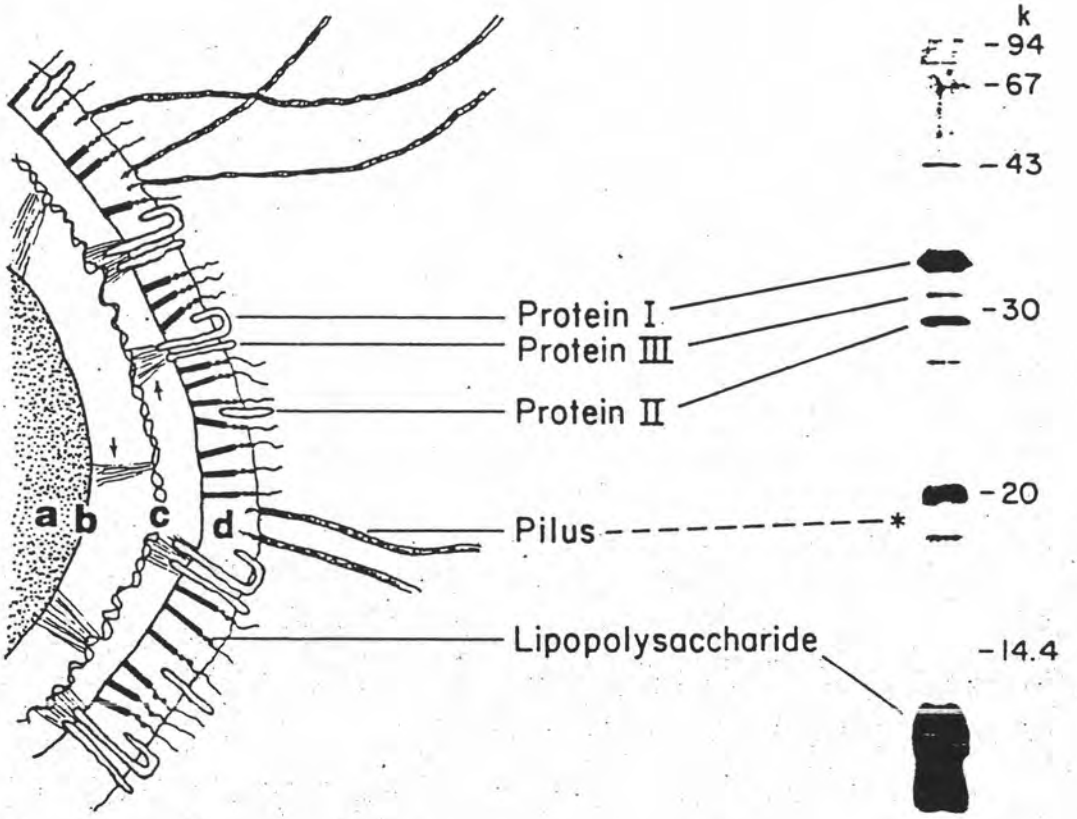


Figure 1 Components of the Surface of the Gonococcus(101). The (a) indicates cytoplasm, (b) inner membrane, (c) peptidoglycan cell wall, and (d) outer membrane.

At the right, these structures are shown on polyacrylamide gel electrophoresis by solubilization of whole gonococci in sodium dodecyl sulfate. The numbers to the right of the gel present the migratory position of known molecular weight markers.

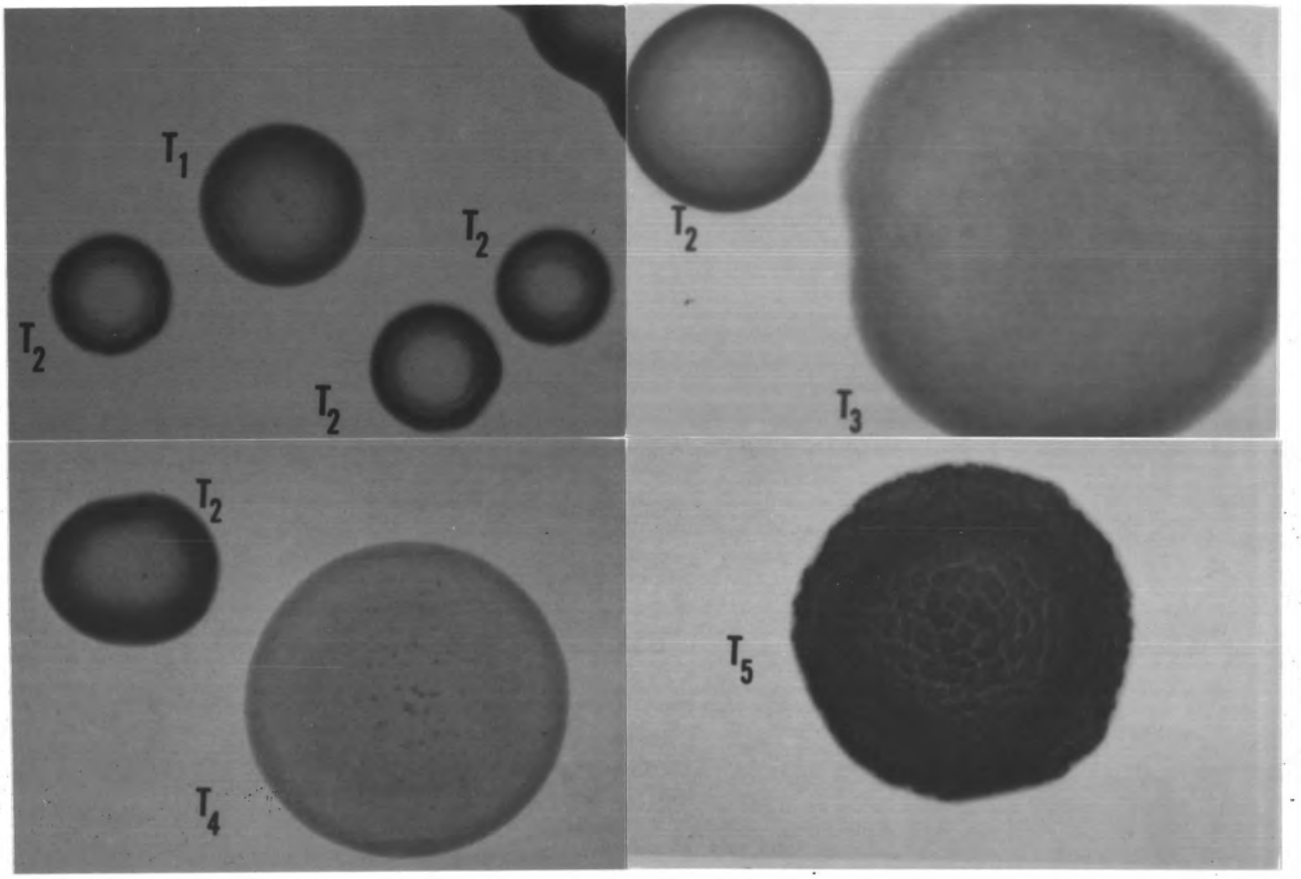


Figure 2 Gonococcal Colony Types 1-5 (original x50).

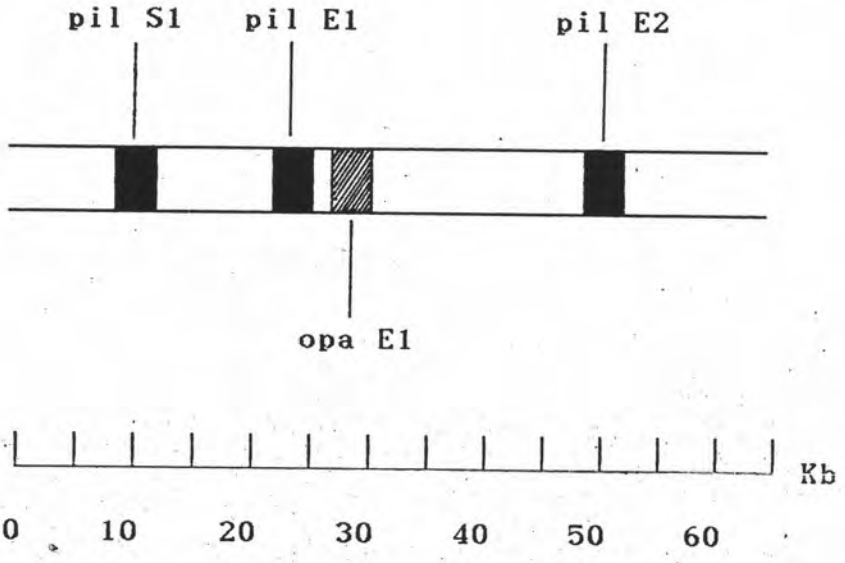


Figure 3 Location of Certain Chromosomal Genes for Pilin or Protein II(127).

The loci pil E1 and pil E2 are transcriptionally active complete pilin structural genes.

The pil S1 is an incomplete and transcriptionally silent pilin locus.

The opa E1 locus is a complete structural gene for one of the proteins II family.

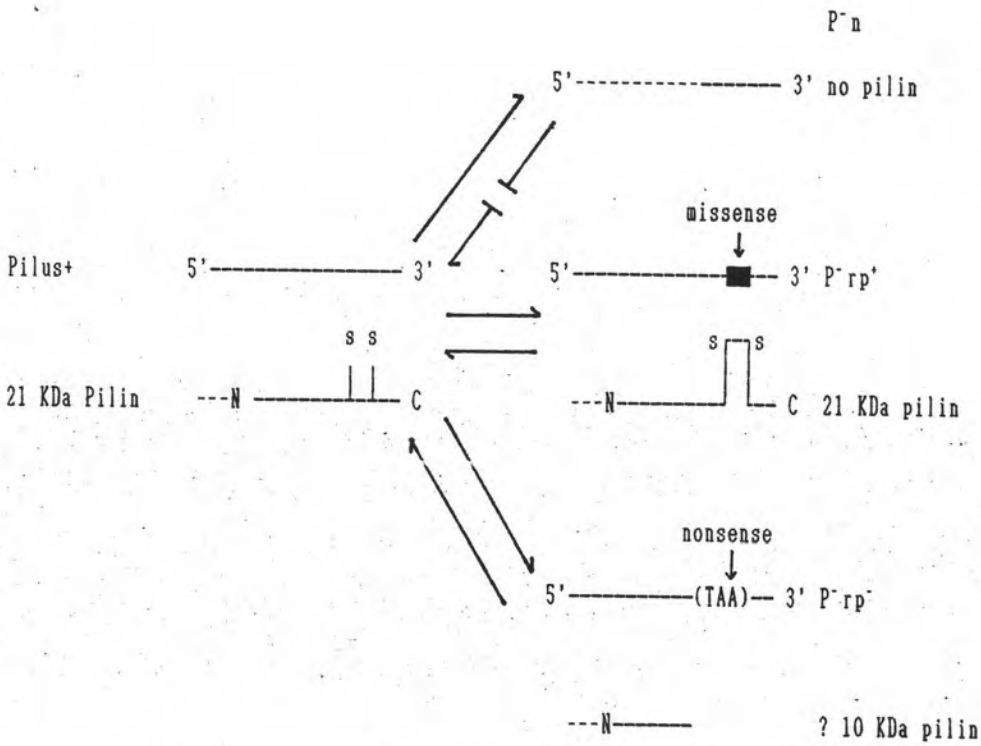
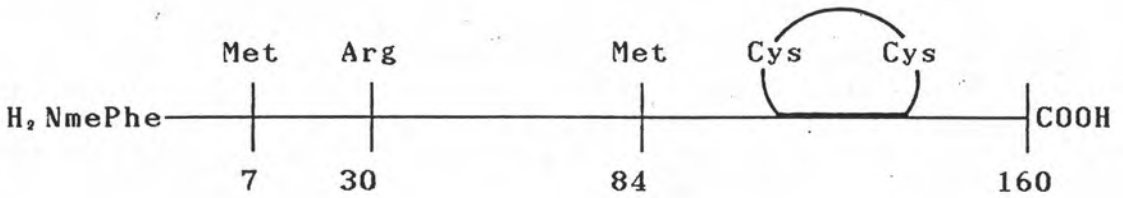


Figure 4 Transitions between Pilus⁺ and Pilus⁻ Phenotypes Described in the Page 22 (126).

Gonococcal Pilin Subunit



Cyanogen Bromide Cleavage

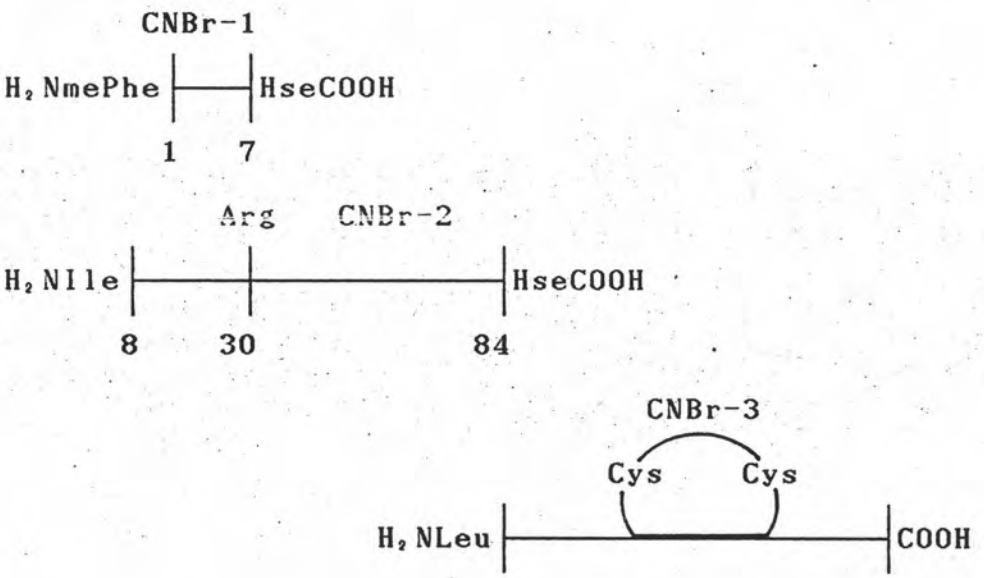


Figure 5 Cyanogen Bromide Cleavage of Pilus Protein (113).

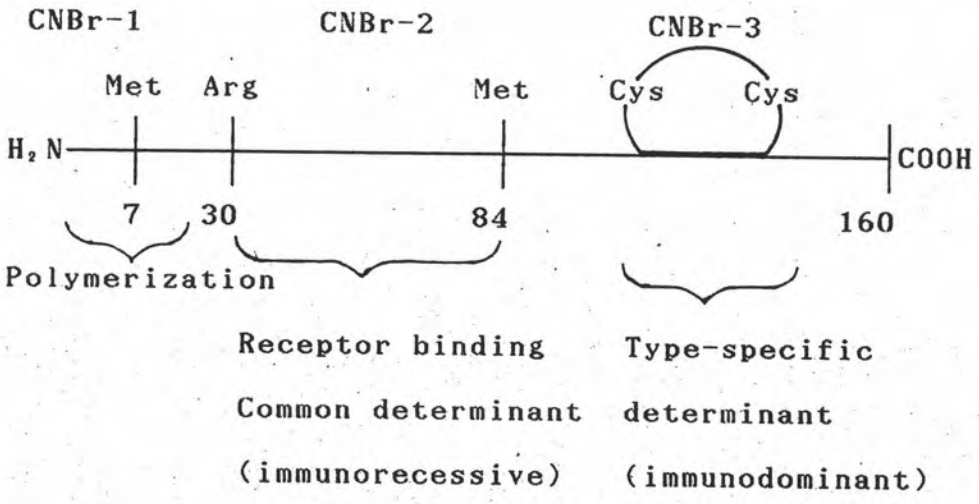


Figure 6 Functional and Antigenic Domains of the Gonococcal Pilus Subunit (113).

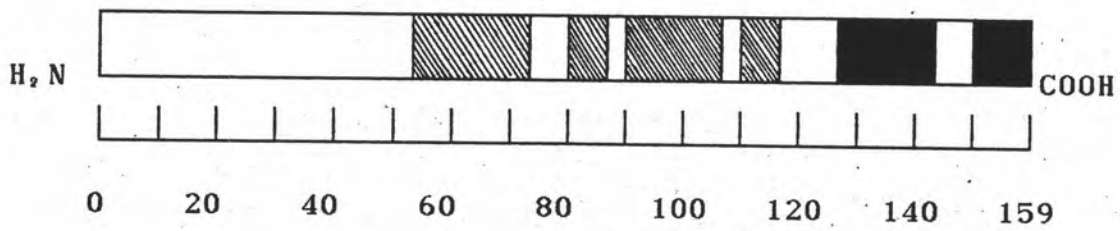


Figure 7 Variability of Pilin Structure as Revealed by Primer Extension DNA sequencing of Expressed pil Genes (127).

White areas are constant regions, hatched areas are semivariable regions, and black areas are hypervariable regions.

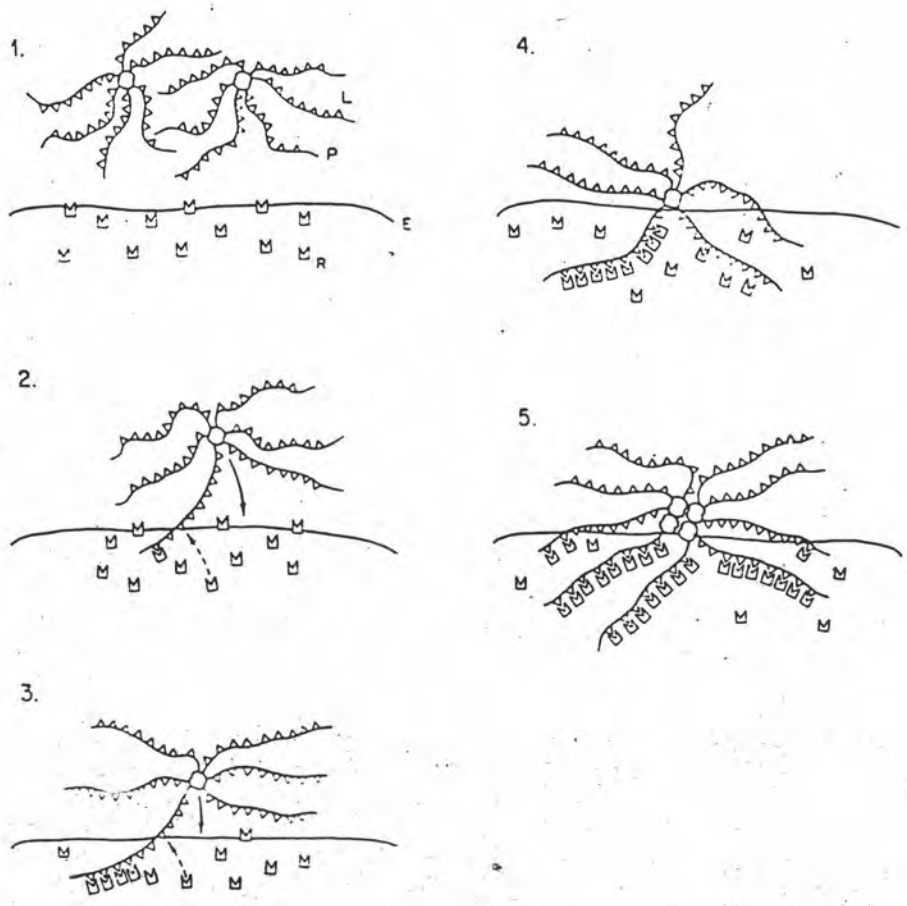


Figure 8 Model of the Adherence of Piliated Bacteria to an Epithelial Cell Surface (120).

The assembly of pilus subunits into the native protein results in a linear array of binding region (L). As bacterium approaches (1) the cell surface (E), a distal subunit binds to a receptor (R) melecule (2). The bacterium is drawn toward the cell surface (3) and (4) as proximal subunits are bound by receptor molecules diffusing in the plane of the cell surface membrane. Once adhesion has occurred, a microcolony may form (5).

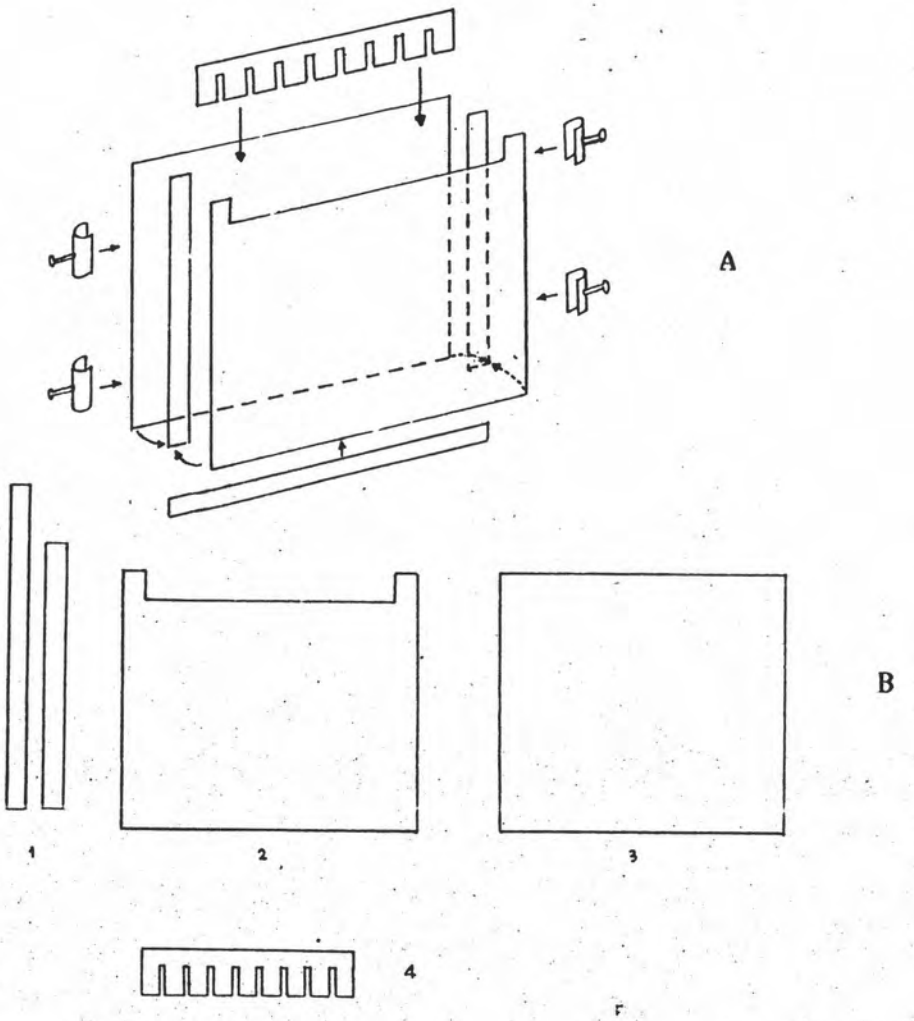
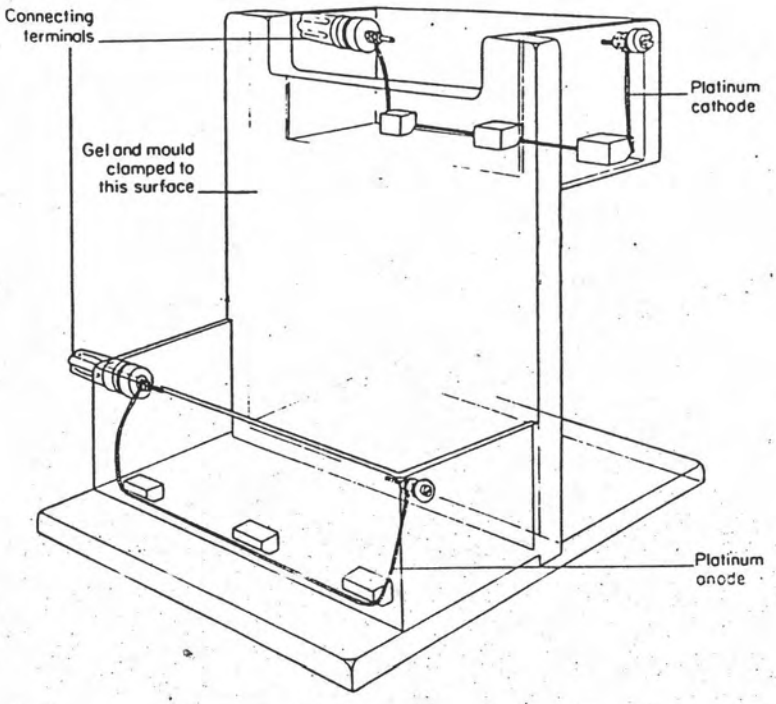


Figure 9 Apparatus for Slab SDS-PAGE.

(A) exploded view of gel mould.

(B) components of the mould. (1) spacers (2) front glass plate (3) back glass plate (4) comb.



C

Figure 10 Apparatus for SDS-PAGE.

(C) electrophoresis tank.

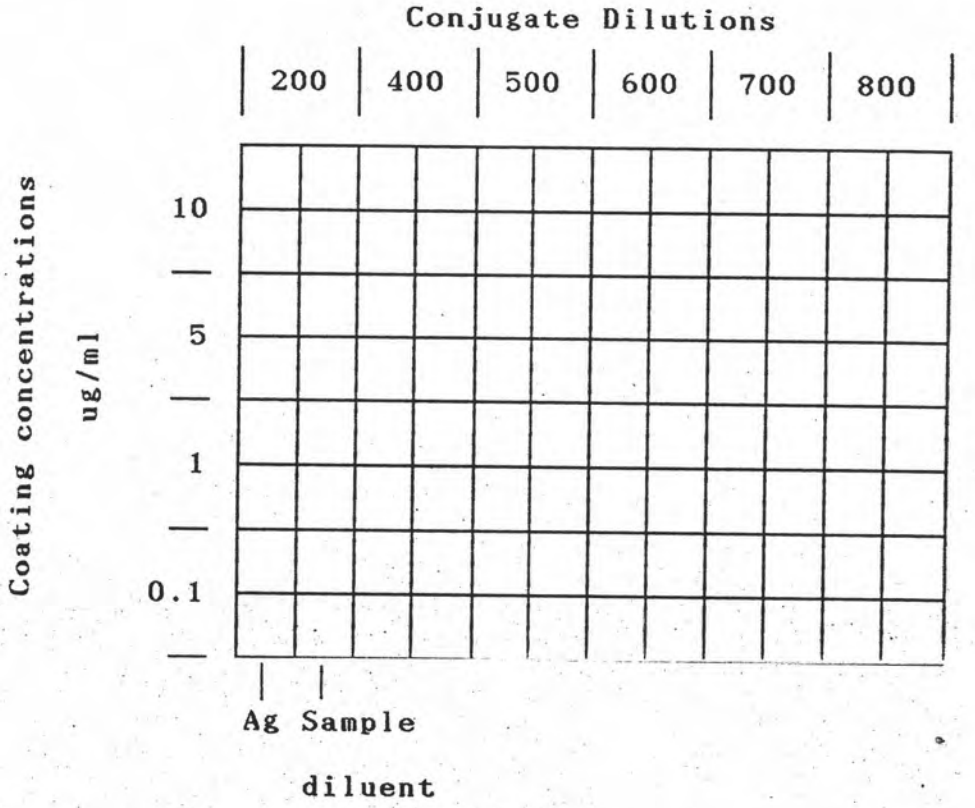


Figure 11 Checkerboard for Determination of Working Dilution of Reagents in the Double Antibody Sandwich ELISA for Pili Antigen.

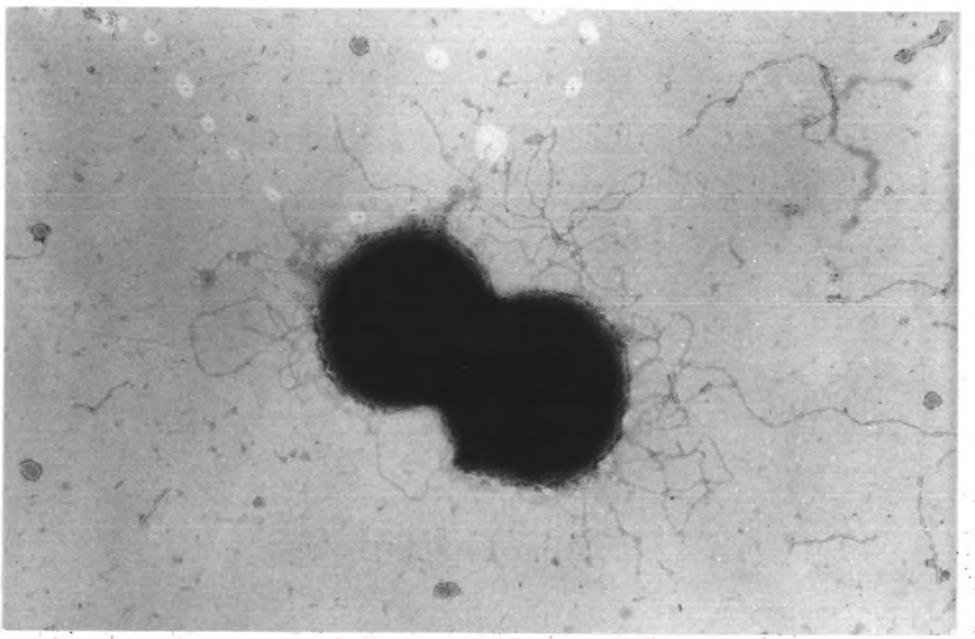


Figure 12 Transmission Electron Micrograph of a Negatively Stained Piliated Gonococcal Isolate Strain S 040629 (type 1). Phosphotungstate x20,000

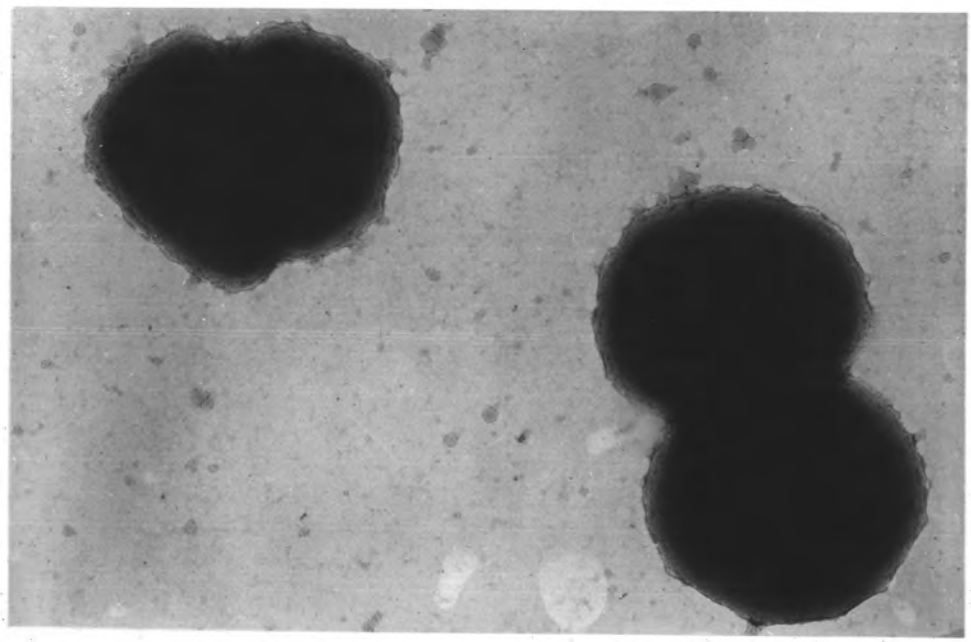


Figure 13 Electron Micrograph of Type 1 Gonococci Strain S 040629 That Had Blended for 2 Minutes.

Phosphotungstate x20,000

Figure 14 SDS-PAGE Analysis of Various Stages of Gonococcal Pili Purification from Strain S 040629.
Lane a,f. standard molecular weight markers, b. supernate after removal of cells at 12,000 xg, 10 min, c. pellet after 12,000 xg, 10 min, d. supernate after 48,000 xg, 60 min, e. pellet obtained after 48,000 xg, 60 min, g. supernate of the centrifugation at 30,000 xg, 10 min, h. pellet of crude first cycle pili obtained from the centrifugation at 30,000 xg, 10 min, i. supernate after 30,000 xg, 10 min (second cycle), j. pellet of purified pili obtained from centrifugation at 30,000 xg, 10 min (second cycle) indicated by arrow.

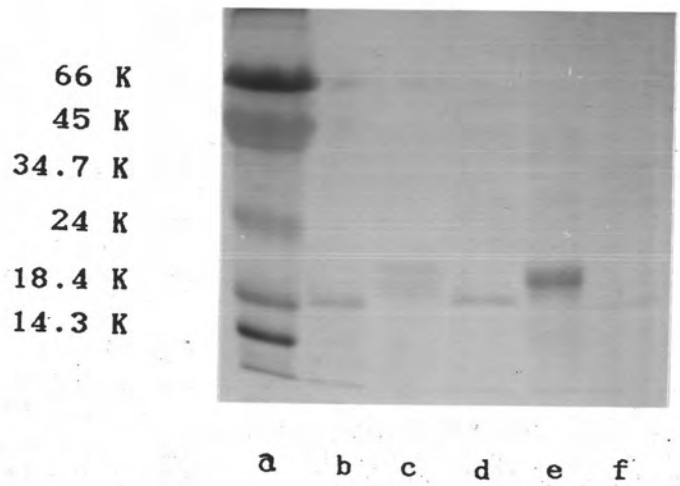
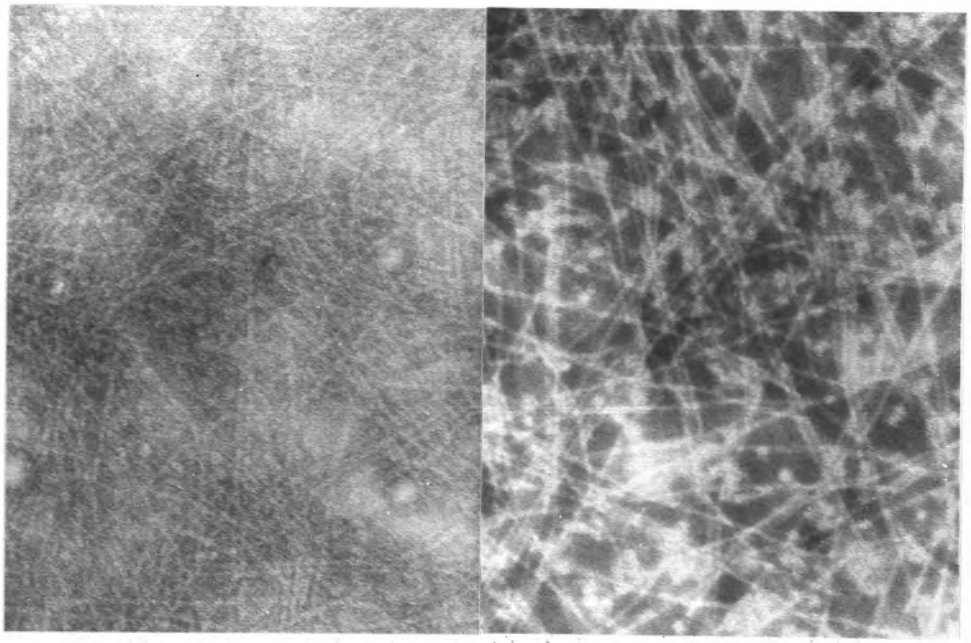


Figure 15 SDS-PAGE of Gonococcal Pili Strains J 070229 (b), S 280229 (c), S 200329 (d), S 040629 (e), S 160829 (f), and Molecular Weight Markers (a).



a b

Figure 16 Electron Micrographs of Purified Gonococcal Pili from Strains S 040629 (a) and S 280229 (b). The pili preparations were stained with 0.5% phosphotungstic acid, pH 7.0. x100,000

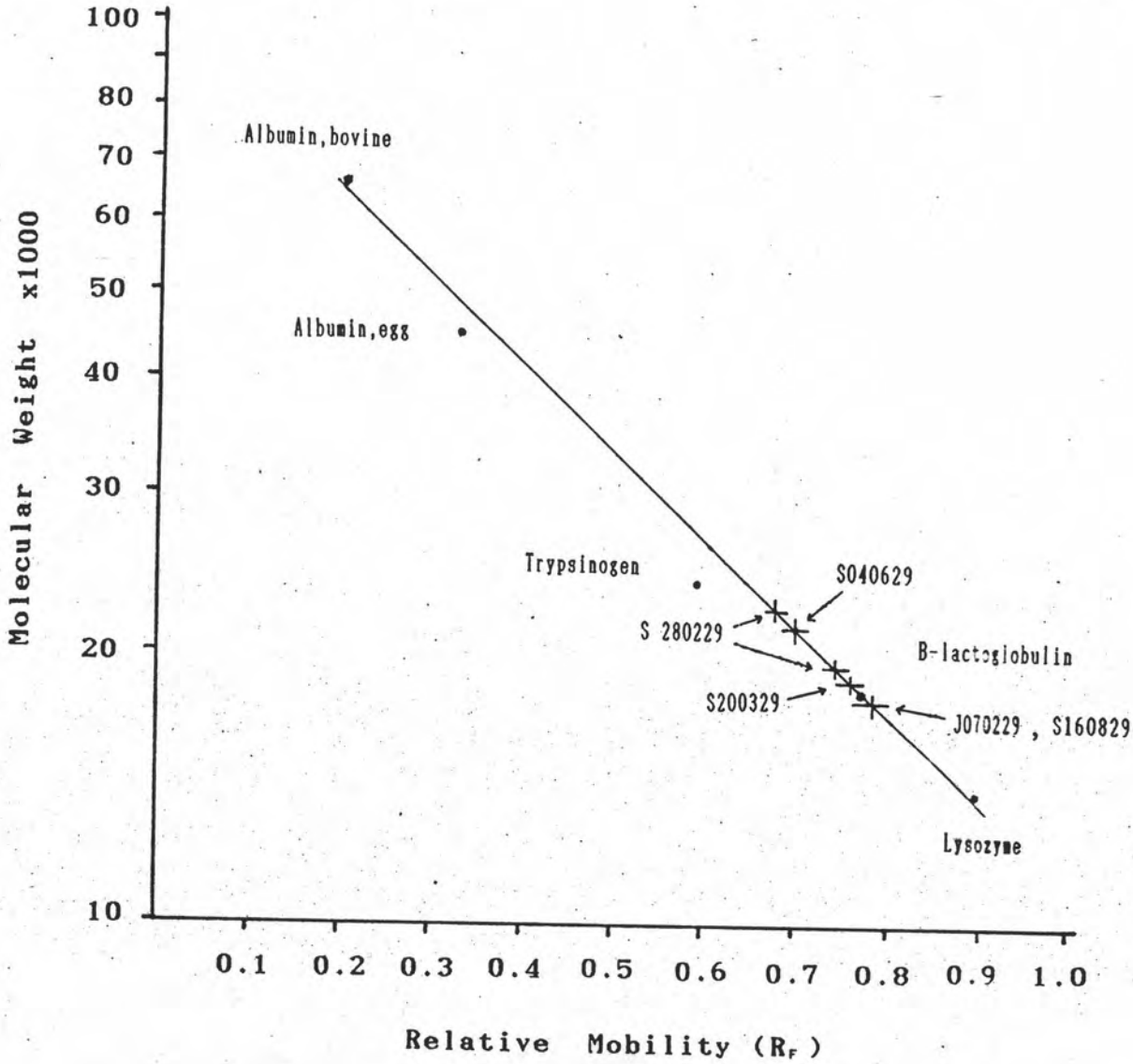


Figure 17 Migration of Gonococcal Pili and Molecular Weight Markers in 12.5% Separating Gel in SDS-PAGE.

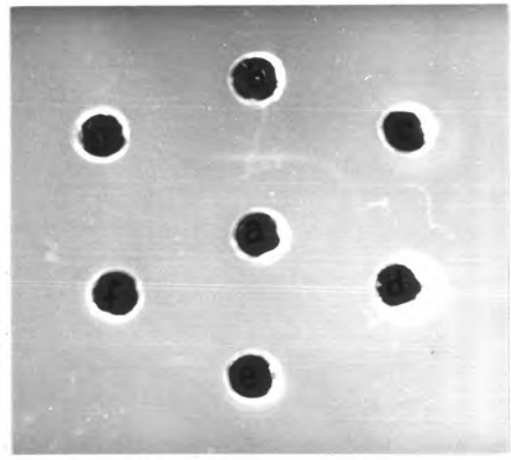


Figure 18 Reaction of Purified Gonococcal Pili S 040629 (Central Well) with Two Fold Serial Dilution of Rabbit Antiserum against Its Pili (Peripheral Wells).
(a): gonococcal pili (b,c,d,e,f,and g): dilution of antiserum initially undiluted, 1:2,1:4,1:8,1:16, and 1:32, respectively.

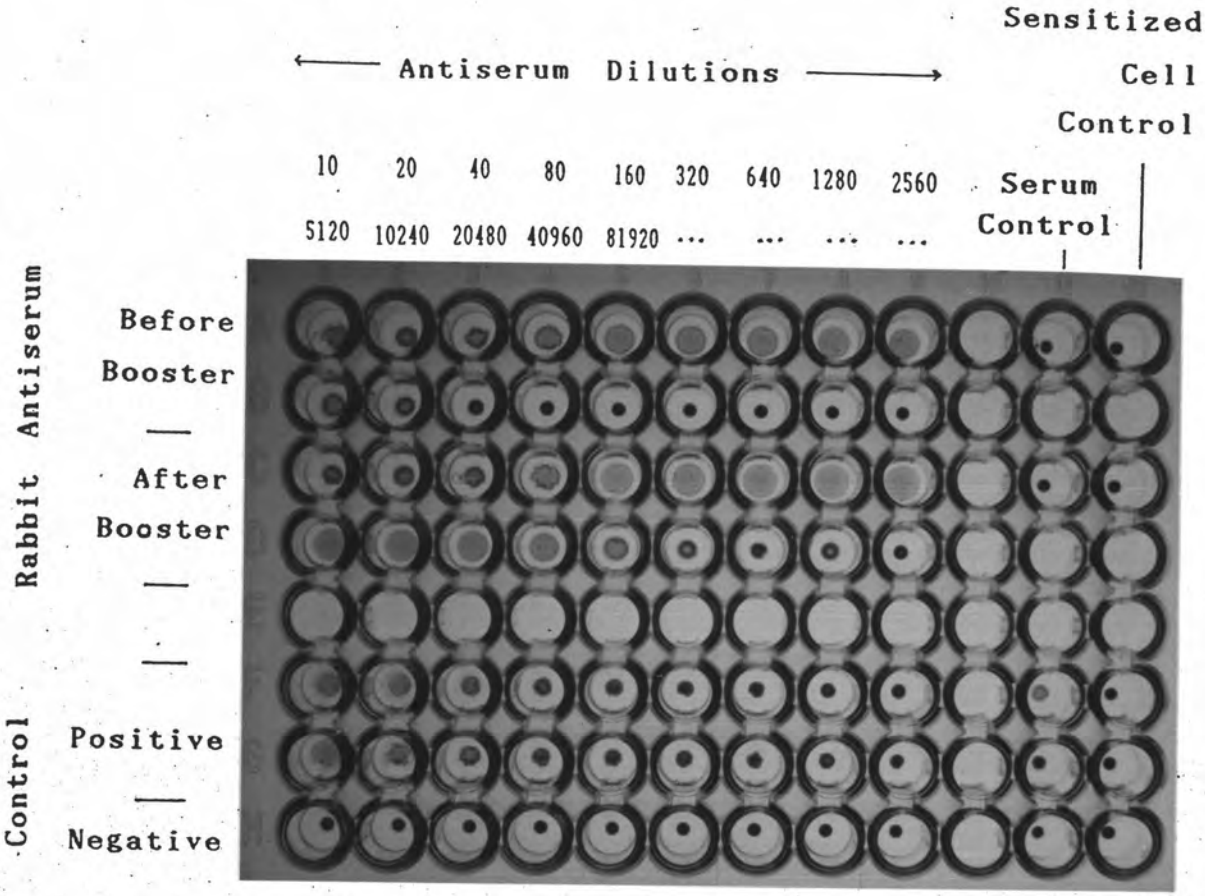


Figure 19 An Indirect Haemagglutination Test for Titration of Rabbit Antigonococcal Pili Antibody.

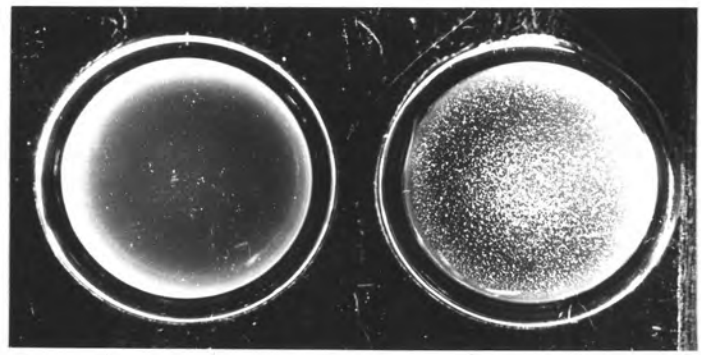


Figure 20 - Typical Coagglutination Test Results with N. gonorrhoeae, Strain S 040629.
Left: control reagent , Right: test reagent.

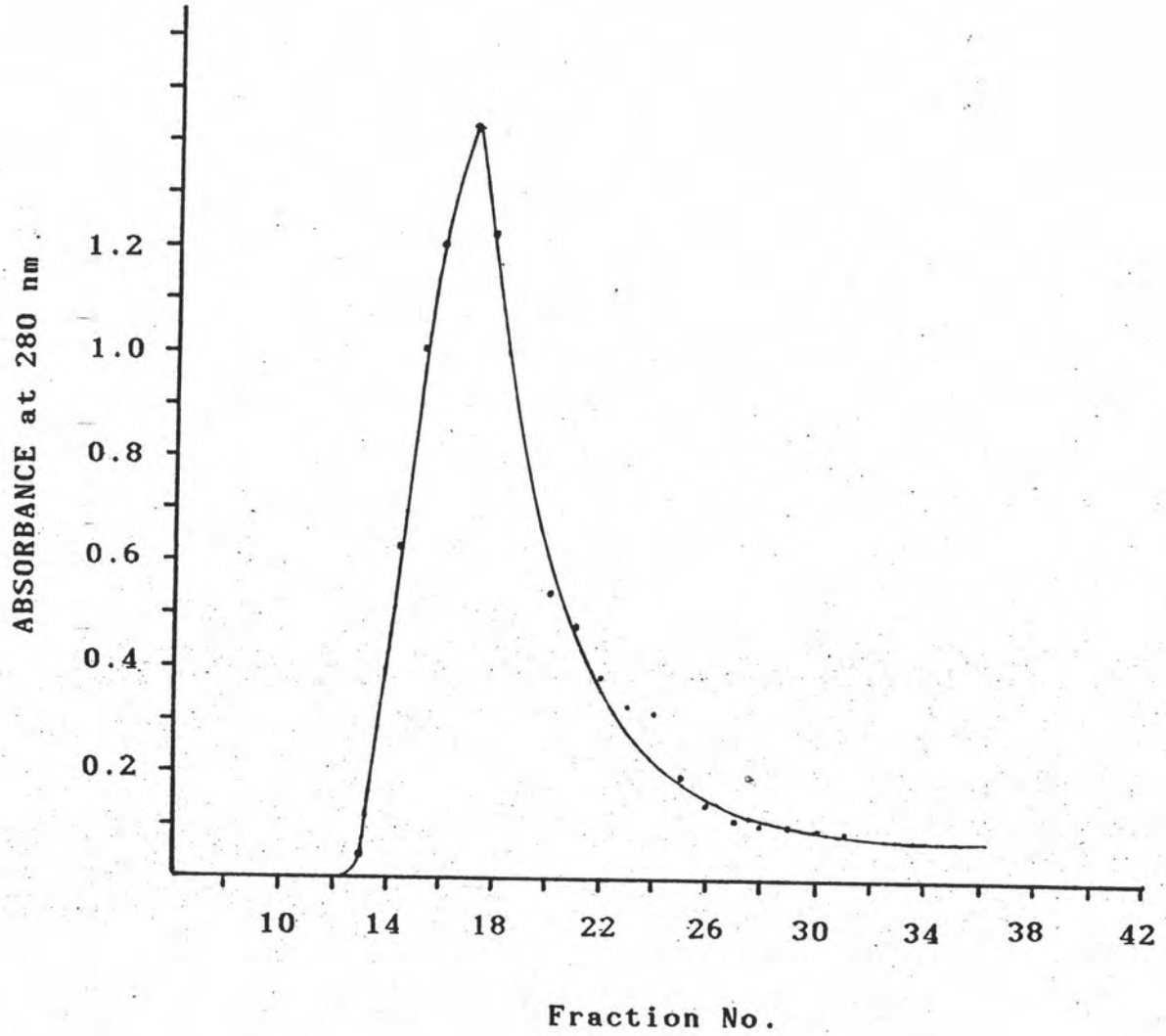


Figure 21 Isolation of IgG from Rabbit Serum on DE-52 Cellulose in 0.01M Potassium Phosphate Buffer pH 8.0.

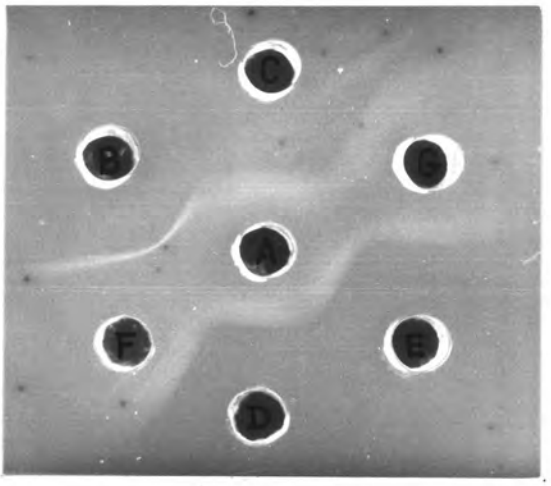


Figure 22 Immunodiffusion of Early Fractions of Isolation of Rabbit IgG.

A:goat anti rabbit IgG

B,C,D,and E:fraction no. 13,14,15,and 16 respectively.

F,G:goat anti rabbit whole serum.

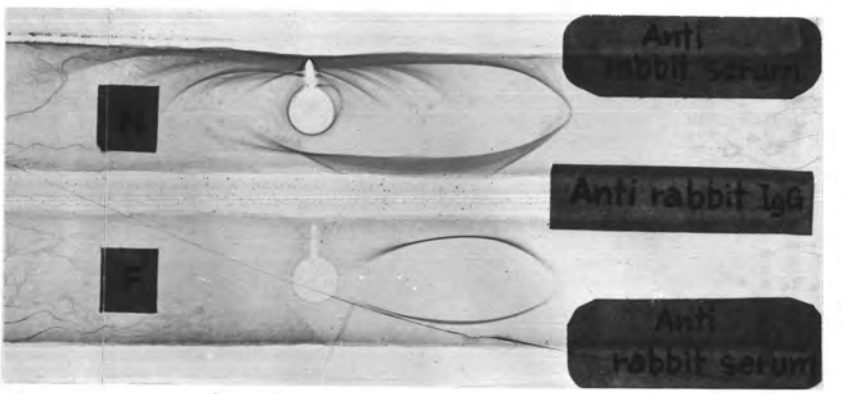


Figure 23 Immunoelectrophoresis of Fraction Containing Purified Rabbit IgG Demonstrating Purity.

N : Normal rabbit serum.

F : Fraction containing rabbit IgG.

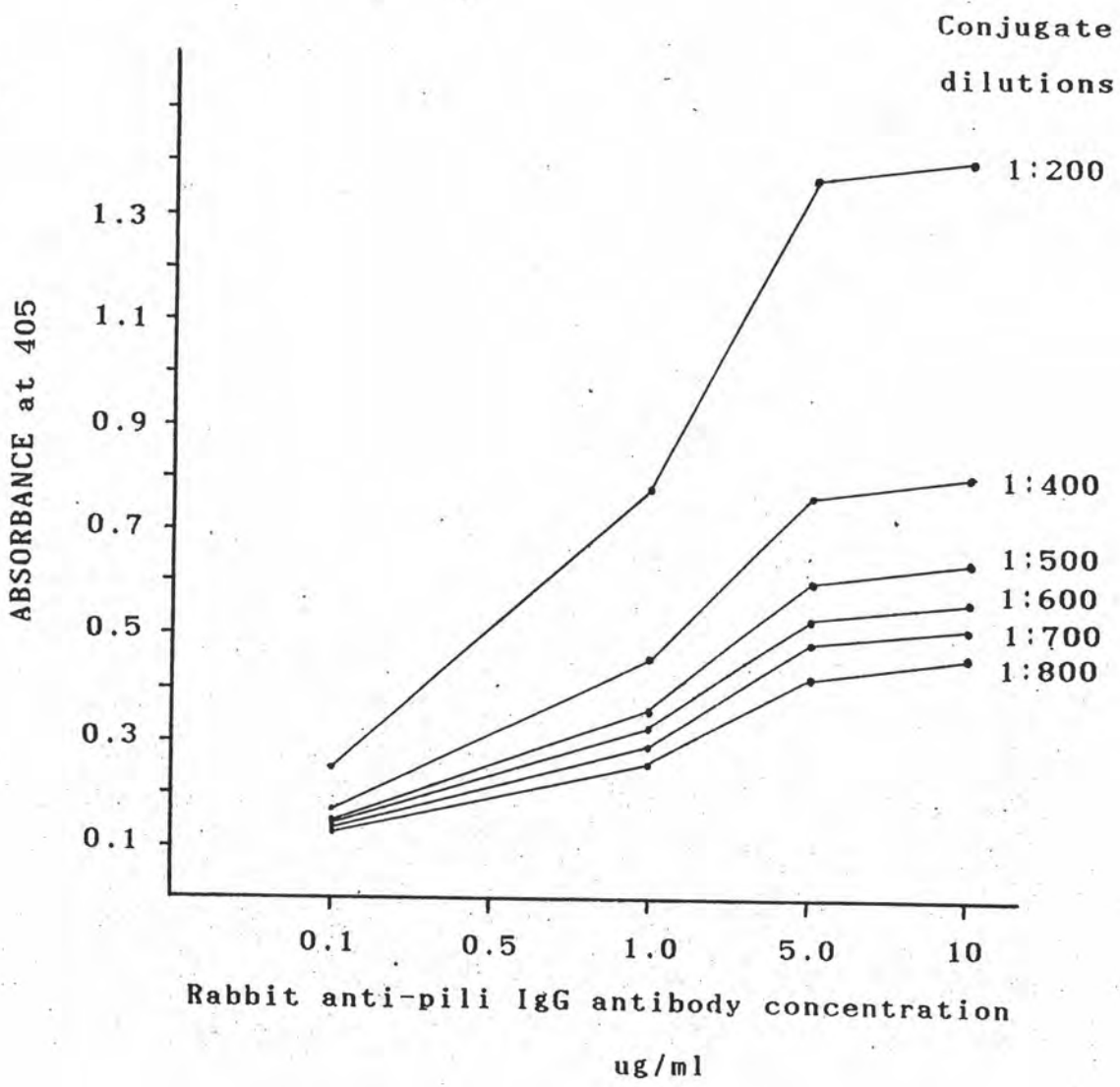


Figure 24 The Optimal Antibody Concentration for Coating ELISA Plate.

Plate was coated with 100 ul of antibody, ranging from 0.1 to 10 ug/ml. Antigen concentration was 5 ug/ml,conjugate dilutions were ranging from 1:200 to 1:800 and each incubation periods was 3 hours,at 37 °C.

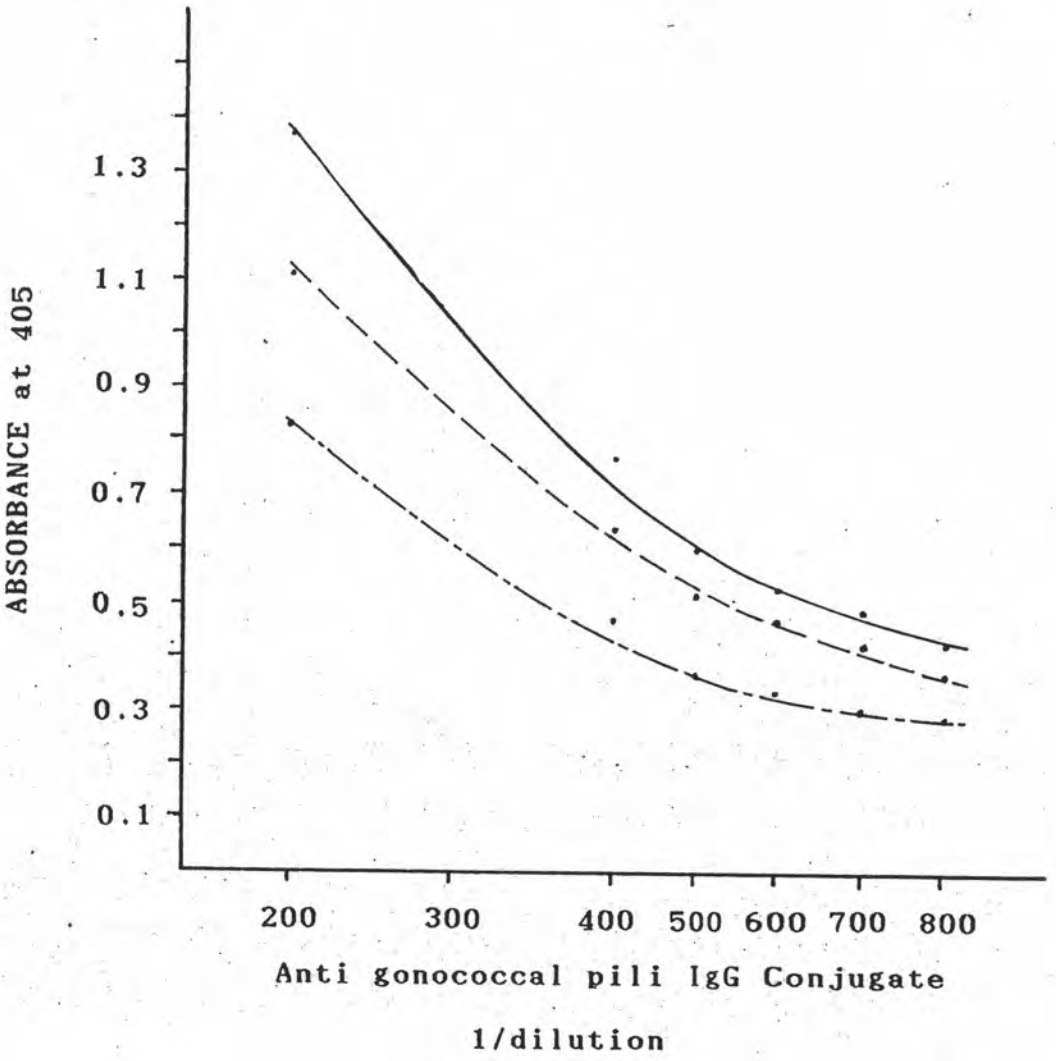


Figure 25 The Optimum of Conjugate Dilution and of Enzyme Substrate Incubation Times.
Plate was coated with 100 ul of antibody, 5ug/ml. Pili antigen concentration was 5 ug/ml, conjugate dilutions were 1:200, 1:400,1:500,1:600,1:700,and 1:800, and each incubation times was 3 hours at 37 ° C. Substrate incubation times were 30 (•—·—•), 45 (•— —•),and 60 (•———•) min ,respectively.

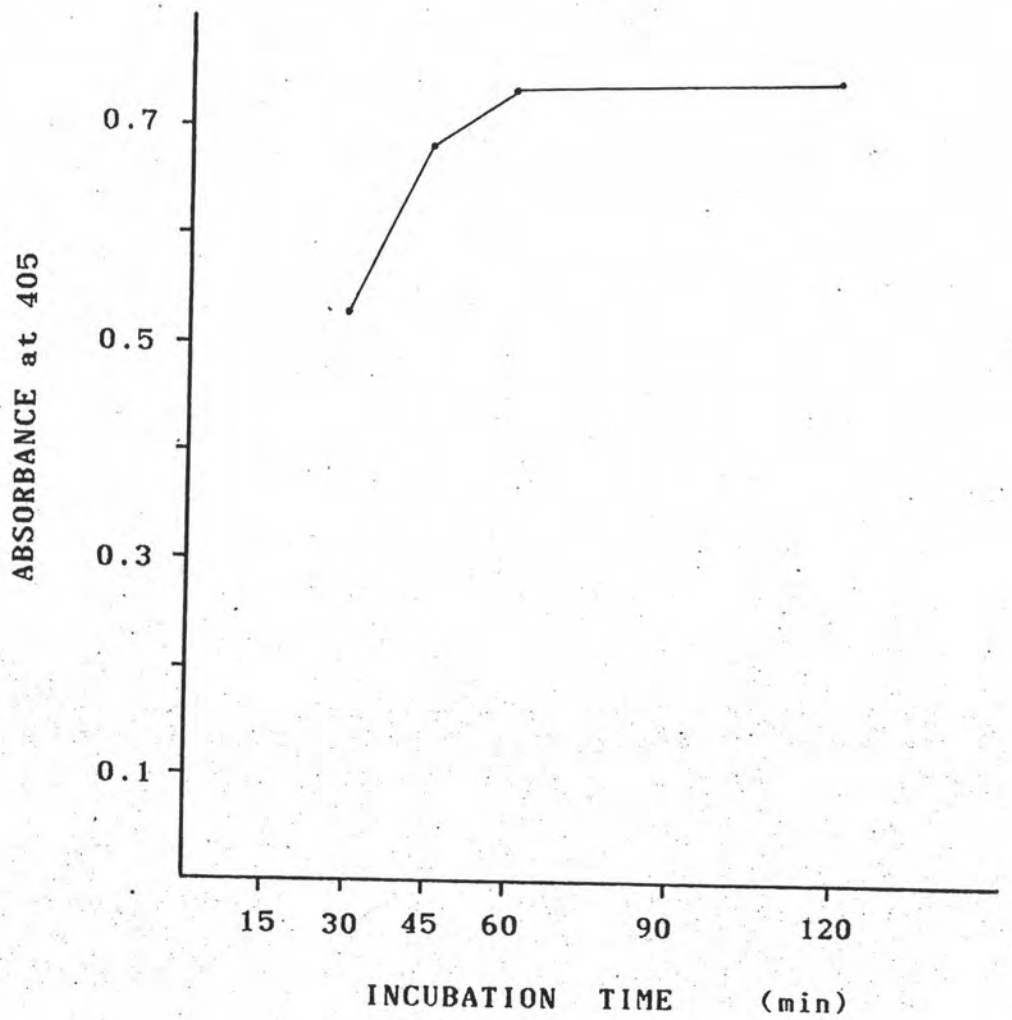


Figure 26 The Optimal Pili Antigen Binding.

Plates were coated with 100 ul of 5 ug/ml antibody. The pili antigen concentration was 5 ug/ml and the incubation period at 37 °C were 30,45,60, and 120 min. Conjugate dilution was 1:300 and incubation time was 120 min at 37 °C.

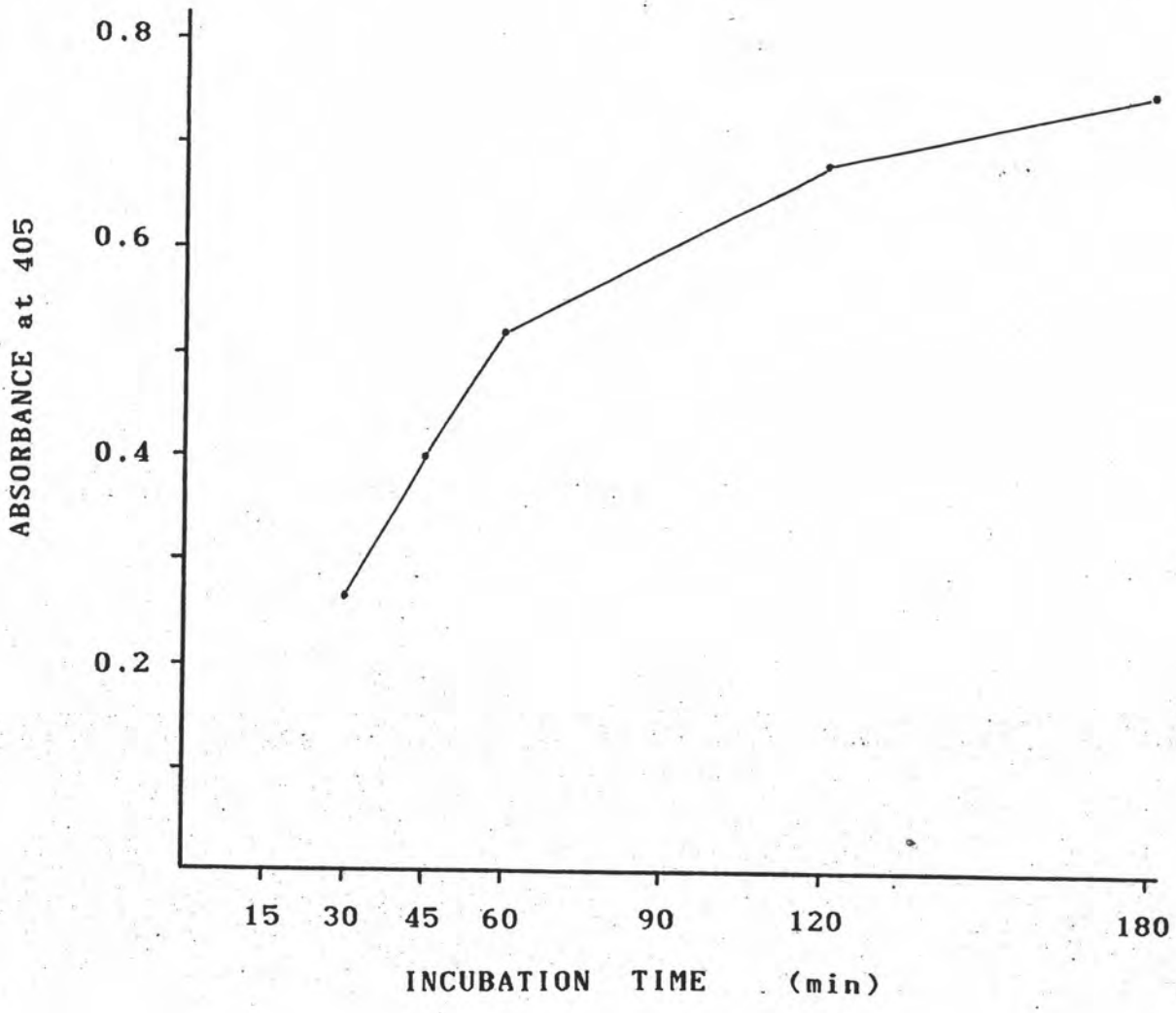


Figure 27 The Optimal Conjugate Binding.

Plates were coated with 100 ul of antibody, 5 ug/ml. The antigen concentration and incubation conditions were 5 ug/ml and 45 min at 37 °C,respectively. Conjugate dilution was 1 in 300 and the incubation periods were 30, 45,60,120,and 180 min at 37 °C.

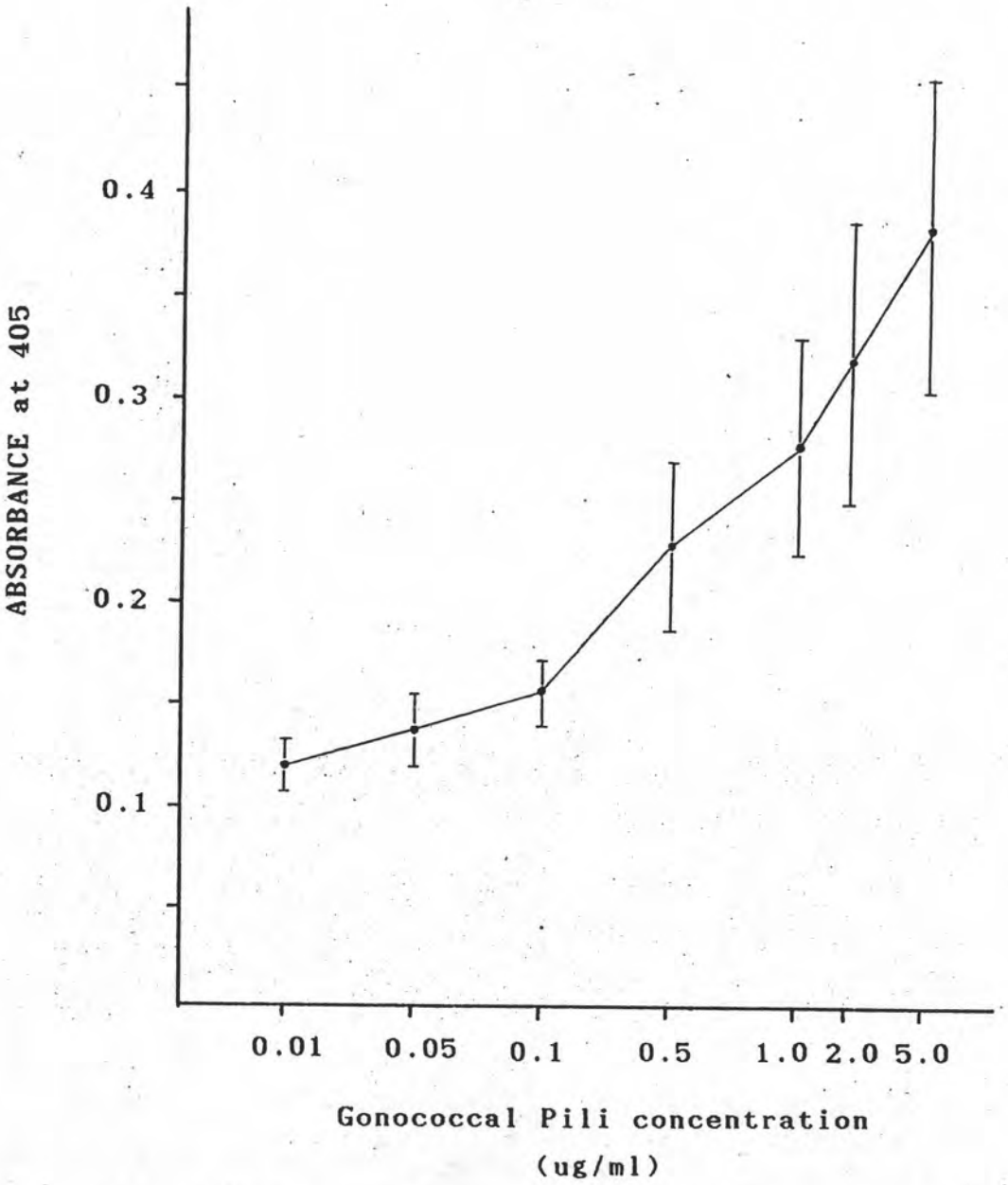


Figure 28 The Typical Curve of the Reference Purified Gonococcal Pili S 040629 Antigen.
Plates were coated with 100 ul of 5 ug/ml antibody. Antigen concentration ranged from 0.01 to 5.0 ug/ml and incubation at 37 °C for 45 min. Conjugate dilution and incubation condition were 1:300 and 45 min at 37 °C, respectively. Substrate incubation was 60 min at 37 °C.

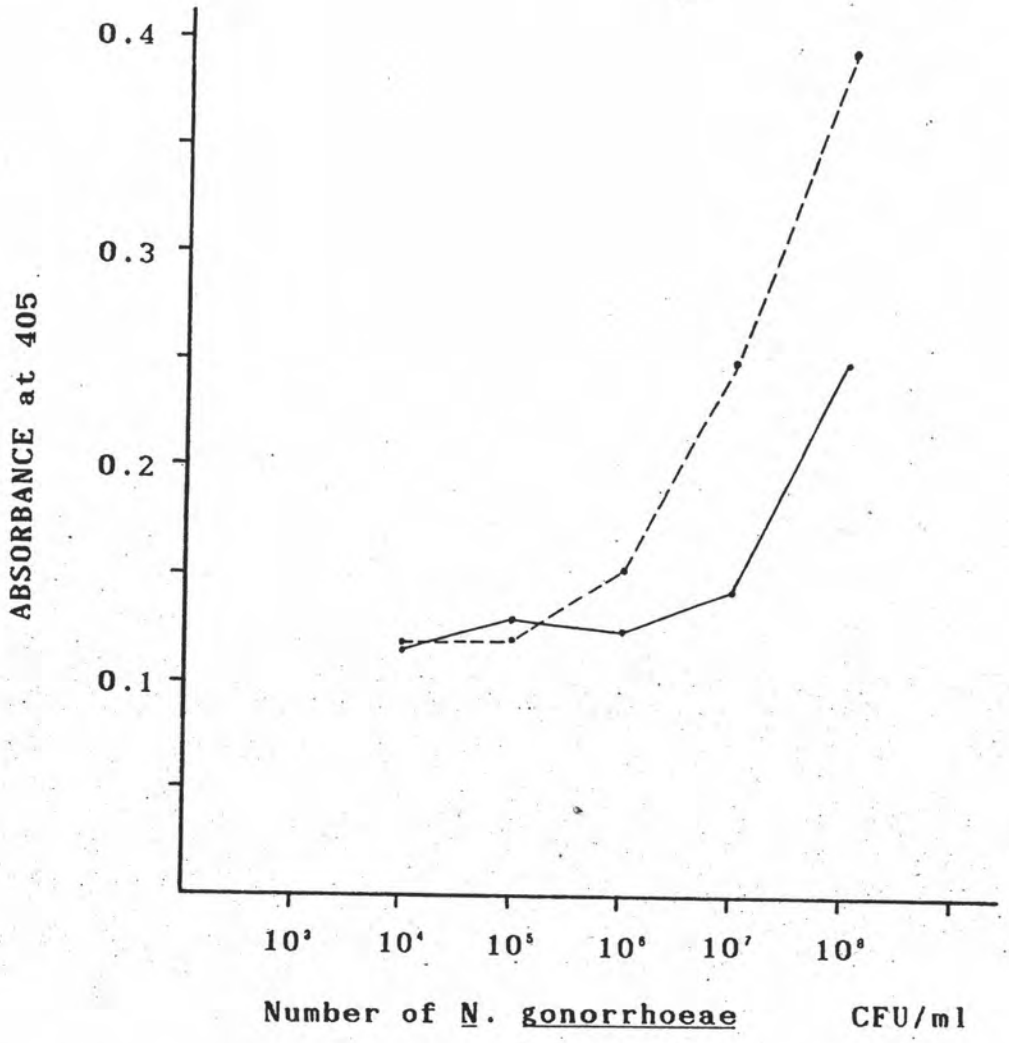


Figure 29 The Comparison of Absorbance Values Obtained by ELISA Using Whole Cells (•——•) and Whole Cell Lysates (•------•) of Gonococcal Strain S 040629.

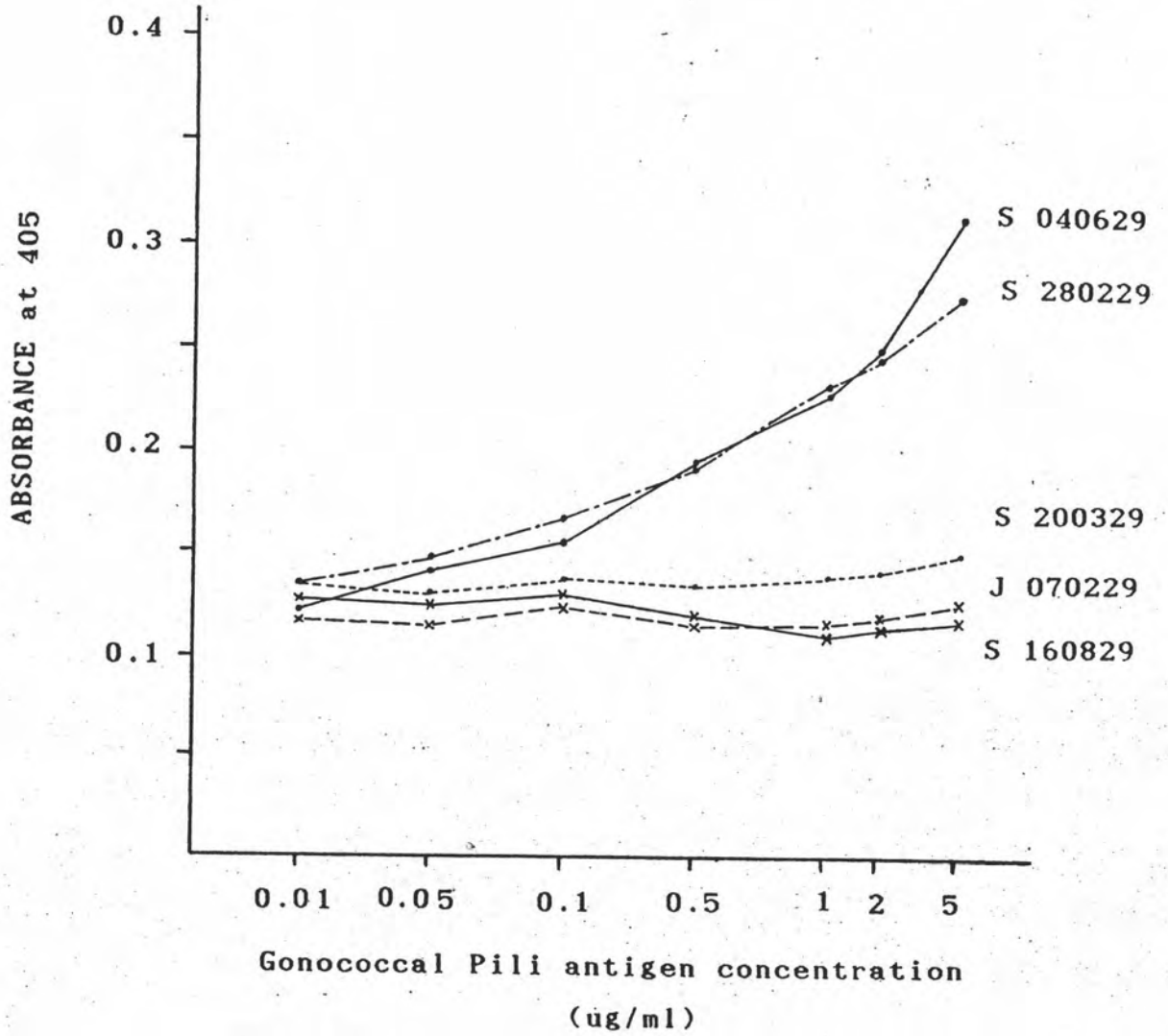


Figure 30 Determination of Cross Reactivity of Rabbit Anti Gonococcal Pili of Different Strains by ELISA.