AN INVESTIGATION OF FACTORS WHICH AFFECT ADHERENCE OF *NEISSERIA GONORRHOEAE* TO HUMAN BUCCAL MUCOSAL CELLS

A Thesis

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Master of Science

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by

D. Scott Primrose

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ABSTRACT

Adherence of Neisseria gonorrhoeae to human buccal epithelial cells was assessed by three methods, radiolabelling of gonococci with ^{14}C or ^{125}I, visual and viable counts. These methods confirmed the adherence of strain 191 type 1 cells but adherence of type 4 organisms was only observed with the radiolabelling procedure. Also adherence of type 1 cells was lower when assessed by visual counting. The viable count assay was shown to be the most suitable method of the three adherence assay methods to study the adherence of gonococci to human buccal cells. With the viable count assay method, type 1 gonococci of six strains varied in their ability to adhere to the host buccal cells in contrast with type 4 organisms all of which did not adhere. Adherence was not significantly different at pH values of 5.2-7.9 and viability of the gonococci was not an important factor. However, adherence depended on time and the gonococci:buccal cell ratio.

A number of potential inhibitors of gonococcus-buccal cell adherence was investigated with the viable count assay. D-glucose, D-mannose, lactose, D-galactose, alpha-methyl-D-galactoside, alpha-methyl-D-glucoside, heparin and N-acetylneuraminic acid did not inhibit the adherence of the 191 type 1 strain. Four lectins, concanavalin A, wheat germ, soybean, and peanut agglutinins, had no inhibitory effect. However, ganglioside (bovine Type III) showed inhibition which was concentration-dependent. This suggests that all or a part of the ganglioside molecule is analogous either to the buccal cell or gonococcal receptor.

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TABLE OF CONTENTS

CHAPTER PAGE

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ATP	Adenosine triphosphate
BBL	Baltimore Biological Laboratories
POPOP	1, 4-Bis(2-(Phenyloxazolyl) Benzene
BMEC	buccal mucosal epithelial cells
CFU	colony-forming units
cpm	counts per minute
cAMP	cyclic Adenosine monophosphate
^{o}C	degrees centigrade
PPO	2, 5-Diphenyloxazole
ESP	enriched single phase
EDTA	ethylenediaminetetraacetic acid
GCBD	Gonococcal Base Defined
g.	grams
g	gravitational unit
HAM	human amnion cells
pI	isoelectric point
LAF	leucocyte association factor
LPS	lipopolysaccharide
µCi	microcurie(s)
MHBSS	modified Hank's balanced salt solution
N	nitrogen
N.S.	not significant
OM	outer membrane
PBS	phosphate buffered saline
PPS	proteose peptone saline
rpm	revolutions per minute
TEM	transmission electron miscroscopy
$(U\text{-}^{14}C)$	uniformly labelled with ^{14}C

CHAPTER I

INTRODUCTION

The adherence of Neisseria gonorrhoeae to various eucaryotic cell types is a recently recognized and studied phenomenon (Swanson, 1973; Ward and Watt, 1972). Since pili were first discovered on virulent type 1 and type 2 cells (Jephcott et al., 1971), they were thought to be responsible for gonococcal adherence. However, recent observations on the adherence of the non-pilated types 3 and 4 suggest that non-pilar surface components are capable of facilitating adherence as well (Tebbutt et al., 1976; James-Holmquest et al., 1974). There has been no positive identification of the surface component or components which facilitate this adherence. One approach to identification of the surface structures involved is through the use of compounds such as carbohydrates, which are known to be present on the surface of the gonococcus and epithelial cell. This could be achieved by attempting to inhibit adherence with these compounds in a competitive situation. It is the aim of this study to investigate several methods of assaying adherence of gonococci to human buccal cells and to study factors affecting adherence in vitro and to make use of carbohydrates and lectins in characterizing those structures on the gonococcus surface which may be involved.

CHAPTER II

LITERATURE REVIEW

Biology of the Gonococcus

Neisseria gonorrhoeae is a nutritionally fastidious, gram-negative, aerobic, diplococcus, first described by Neisser in 1879 and first cultured on artificial medium in 1882 (Cited by Davis *et al.*, 1973).

The various colonial morphologies displayed by this species have been well characterized (Kellogg *et al.*, 1963, 1968; Jephcott and Reyn, 1971; Reyn *et al.*, 1971; Chan and Wiseman, 1975). Although only colonial types 1, 2 and 4 have been dealt with in this study, types 1', 3 and 5 are included in Table I for the sake of comparison. Colony types 1 and/or 2 are more typically seen upon primary subculture of appropriate specimens taken from infected hosts, whereas types 1', 3, 4, and 5 are more commonly derived from further passages *in vitro*. It has also been observed that within colonial types color and hence opacity can vary greatly (Brown *et al.*, 1974; Jephcott and Reyn, 1971; Diena *et al.*, 1974; Swanson, 1977; James and Swanson, 1978 *b*; Salit *et al.*, 1978). According to Swanson (1977, 1978), variation in colony coloration and opacity is not due to a chromophoric metal-containing protein. However, the darker color does appear to be associated with the presence of proteins with molecular weights varying from 24,000-30,000 daltons. These proteins seem to promote gonococcus-to-gonococcus adherence thereby affecting gonococcal growth. This may be responsible for the darker colored colonies. Gonococci from lightly colored colonies may possess traces of these proteins or lack them entirely (Walstad

TABLE I

CHARACTERISTICS OF THE COLONIAL MORPHOLOGIES OF N. GONORRHOEAE

Type	Size (mm)	Color[a]	Elevation	Edge	Opacity[b]	Structure	Reference[c]
1	0.5	d.g.	convex	entire	tl	amorphous	1.
1'	0.7	v.d.g.	convex	crenated	op	granular	2.
2	0.5	d.g.	convex	entire	tl	amorphous	1.
3	1.0	l.b.	low convex	entire	tl	granular	1.
4	1.0	c.	low convex	entire	tp	amorphous	1.
5	1.4	d.b.	convex	crenated	op	granular	2., 3.

a. d.g.(dark gold); l.b.(light brown); c.(colorless); v.d.g.(very dark gold); d.b.(dark brown).

b. tl(translucent); tp(transparent); op(opaque).

c. 1. Kellogg et al. (1963,1968).

2. Chan and Wiseman (1975).

3. Jephcott and Reyn (1971).

et al., 1977). In contrast to gonococci from transparent or lighter colonies those from opaque or darker colonies are aggregated and trypsin sensitive. Watt et al. (1978) have shown that gonococci which lacked a surface protein with a molecular weight of 29,000 daltons adhere as well to mucosal cells as gonococci possessing this protein. If this protein is one of those associated with colony color and opacity then this evidence suggests that adherence of gonococci is eucaryotic cells is independent of these proteins.

Cell Envelope of Gram-negative Bacteria

As shown in Figure 1 the typical envelope of Gram-negative bacteria includes an inner cytoplasmic membrane followed by a periplasmic space containing a thin rigid peptidoglycan layer. These structures are surrounded by an outer trilamellar membrane, invariably containing lipopolysaccharide (LPS). Capsules, pili and flagella may also be found in Gram-negative bacteria (Costerton et al., 1974).

The cell envelope of the gonococcus fits the above description but does not possess flagella (Swanson, 1972; Novotny et al., 1975; Wolf-Watz et al., 1976). There is some evidence which suggests that the gonococcus may possess a capsule (see page 8). Wolf-Watz et al. (1976) have compared the outer membrane (OM) of E. coli with that of the gonococcus. In contrast to E. coli, the OM of the gonococcus was found to be unattached to the peptidoglycan layer, more permeable to gentian violet and showed a low lipase activity. There were fewer proteins in the OM of the gonococcus and they were more hydrophilic than those of E. coli. Similarities in the OM of the two organisms include density, LPS carbohydrates, fatty acids and phospholipids.

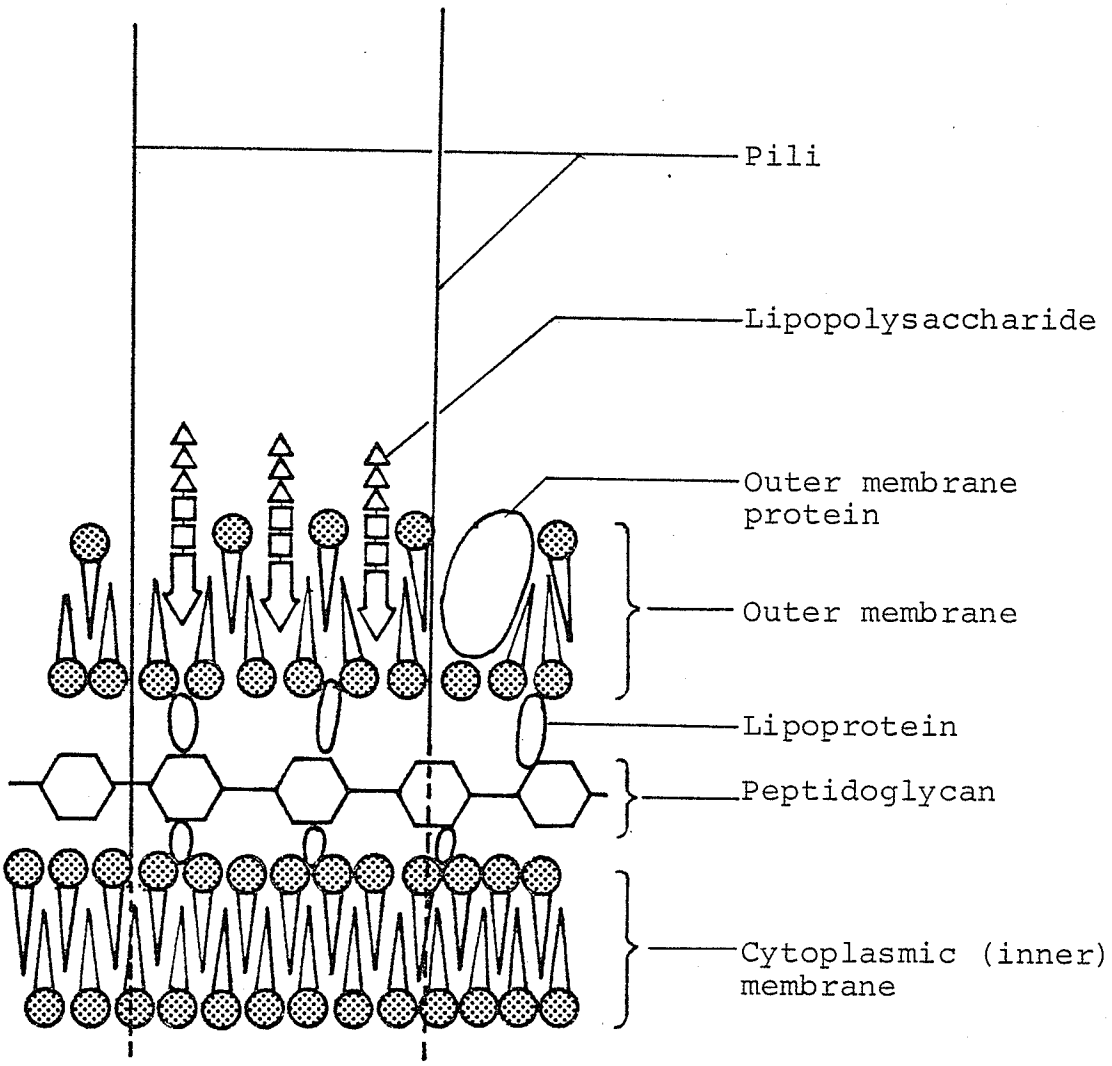

Figure 1. Gram-negative bacterial cell envelope.

Gonococcal Surface Components

Pili: Gonococci from colony types 1 and 2, but not 3 or 4, were found to possess pili (Jephcott et al., 1971; Swanson et al.,1971) and it has been demonstrated that they are antigenically heterogeneous (Novotny et al., 1975; Buchanan, 1975). No electron microscopically visible pili could be detected on cells from colony types 3 and 4 and Buchanan (1978) could detect no non-filamentous pilus antigen in colony type 4 gonococci from three strains, using an enzyme-linked immunosorbent assay. Gonococcal pili are 7-8 nanometers in diameter and can vary in length from 2-4 microns (Swanson et al., 1971); and are composed of protein subunits of varying molecular weights (16,000-24,000 daltons) (Buchanan et al., 1977: Pearce et al., 1978; Hermodson et al., 1978). Some gonococcal pili, apparently, have some hexose residues and phosphate groups associated with their subunits (Robertson et al., 1977). An unusual terminal amino acid, N-methylphenylalanine, and a homologous, N-terminal, hydrophobic sequence have been found in antigenically dissimilar pili preparations from four strains of gonococci (Hermodson et al., 1978). Pili have been correlated with gonococcal virulence and have been implicated in attachment to erythrocytes (Punsalang and Sawyer, 1973: Koransky et al., 1975; Waitkins, 1974), leucocytes (Blake et al., 1975), and other eucaryotic cells (Swanson, 1973; James-Holmquest et al., 1974; Tebbutt et al., 1976; Mardh and Westrom, 1976; Tramont and Wilson, 1977; Pearce and Buchanan, 1978). Although types 1 and 2 gonococci grown in vitro possess pili the question still arises concerning in vivo pilus production and its significance (James and Swanson, 1978 a; Evans, 1978).

Outer membrane proteins: The outer membrane protein composition of the gonococcus is relatively simple and less complex than that of E. coli (Wolf-Watz et al., 1976; Johnston et al., 1974). Three major proteins have been identified in isolated outer membranes of gonococci. These have molecular weights of 34,500, 22,000 and 11,500 daltons (Johnston et al., 1974). Similar findings were obtained by Wolf-Watz (1975) with their predominating protein having a molecular weight of 35,000 daltons. The function of the 34,500 dalton "principal outer membrane protein" has not been elucidated, although involvement in resistance to serum bactericidal activity has been shown (Hildebrandt et al., 1978). It also seems to be present in all gonococcal strains, although ranging in molecular weight from 32,000-39,000 daltons (Johnston et al., 1976; Hildebrandt et al., 1978; Heckels, 1977, 1978; Swanson, 1978). The second predominant protein (22,000 daltons) also appears to vary in molecular weight and may or may not be present (Heckels, 1978; Lamden and Heckels, 1979). A third major outer membrane protein (Protein III) has been shown by Heckels (1978) to be located on the inner surface of the outer membrane and was present in all gonococcal strains tested. The function of this protein is not yet known. Although the functions of the gonococcal outer membrane proteins are not well defined, indications are that they may be important in gonococcal interaction with eucaryotic cells, with the leucocyte association factor (LAF) serving as an example of this (Swanson, 1977).

Lipopolysaccharide moiety: Components of LPS have been identified through analysis of purified LPS of gonococci from various colony types (Maeland, 1969; Perry et al., 1975; Stead

et al., 1975; Wolf-Watz et al., 1975; Wiseman and Caird, 1977). Glucose galactose, glucosamine, heptose, 2-keto-3-deoxyoctonic acid (KDO), phosphate and Lipid A are commonly observed constituents. Rhamnose, xylose, fucose, galactosamine, mannose and N-acetylneuraminic acid are other saccharides reported to be present, variably, in gonococcal LPS (Perry et al., 1975; Wiseman and Caird, 1977), suggesting the presence of additional side chains attached to the core oligosaccharide. The LPS has been implicated in adherence to eucaryotic cells (Watt et al., 1978). It is known that bacterial polysaccharides can be involved in this interaction (Costerton et al., 1977; Gibbons and van Houte, 1975; Jones, 1977; Freiner et al., 1978). Lipopolysaccharide receptors have been shown to be present on human erythrocytes and leucocytes (Springer et al., 1973).

Capsule: Some evidence based on light and electron microscopy suggests the presence of a capsule-like external layer on the gonococcus, composed of polysaccharide material, which can be present in both in vitro and in vivo-grown gonococci (Hendley et al., 1977; James and Swanson, 1977; Richardson and Sadoff, 1977; Demarco de Hormaeche et al., 1978). The quality and quantity of this layer seems to be strain-dependent and its presence seems to depend on the culture medium (Hendley et al., 1977; Richardson and Sadoff, 1977).

With respect to eucaryotic cell association of gonococci, the capsule appears to be correlated with inhibition of phagocytosis by human leucocytes (Richardson and Sadoff, 1977; Hendley et al., 1977) and there is evidence which shows that an encapsulated variant of the P9 strain adhered less well to human buccal cells and HEp-2 cells than the non-encapsulated

form. This decreased attachment of encapsulated gonococci to epithelial cells may be due to blocking or masking of some of the gonococcal surface adhesins by the capsular material.

Adherence of Bacteria to Eucaryotic Cells

Bacteria associated with animals, whether normal flora or non-indigenous flora, exhibit specific surface interaction with animal cells, leading to attachment. Although a number of the bacterial surface components which mediate this adherence have been identified in a few cases, the majority of bacteria including N. gonorrhoeae have not had their attaching antigens or adhesins thoroughly examined as yet.

Studies with E. coli have indicated that this organism can attach to gastrointestinal and urinary tract epithelium. One or more surface components may facilitate this attachment including pili, LPS, K-antigen-associated components and a mannose-specific surface protein (Salit and Gotschlich, 1977; Fowler and Stamey, 1977; Ofek et al., 1977; Smith, 1977; Svanborg-Eden, 1978; Eshdat et al., 1978; Schaeffer et al., 1979). E. coli, however, is not unique in its possession of multiple adhesins as many other organisms appear to possess several attaching mechanisms and possible receptor sites on the host cell. Examples include Streptococcus mutans which has an undefined, primary interaction with tooth enamel and has a secondary, glucan-mediated attachment system (Jones, 1977). Streptococcus pyogenes appears to attach via both M-protein and lipoteichoic acid (Gibbons and van Houte, 1975; Beachey and Ofek, 1976; Alkan et al., 1977). Vibrios, commensal oral Streptococcus species, corynebacteria, human indigenous enteric

organisms, lactobacilli and Bordetella apparently possess several adhesins and agglutinins which are important in epithelial adherence (Savage, 1972; Jones, 1977).

The subject of this investigation, N. gonorrhoeae, also appears to have several surface components which are directly involved in adherence to eucaryotic cells (Smith, 1977; Ward and Watt, 1977; Swanson, 1977; Jones, 1977).

Adherence of N. gonorrhoeae to Eucaryotic Cells

Ward and Watt (1972) studied human urethral cells of males infected with N. gonorrhoeae using transmission electron microscopy (TEM) which demonstrated apparent diplococcal forms closely associated with the membranes of the epithelial cells and mucous-secreting cells. Some of the gonococcal cells (diplococcal forms) can be seen by TEM deeply embedded in the host cells. This suggests that random association, resulting from preparation of specimens for TEM, was not solely responsible for the intimate contact of gonococci and host urethral cells. This implied that an attachment mechanism was responsible for the interaction.

Swanson (1973) reported that pilated type 2 gonococci associated to a much greater extent with suspensions and monolayers of human amnion cells (HAM) than did the non-pilated type 4 cells. The latter exhibited little adherence whereas the former's adherence, apparently, was mediated by the pili. On occasion HAM cells were found to contain intracellular gonococci surrounded by "membrane-limited" vesicles, suggesting that ingestion had occurred after the gonococci had attached.

Waitkins and Flynn (1973) have grown types 1 and 4 gonococci in cultures of mouse fibroblast cells, Vero monkey-kidney cells

and LLCMK2 (derived) monkey-kidney cells and electron micrographs, like those of Ward and Watt (1972), show evidence of attachment with ingestion of gonococci.

James-Holmquest et al. (1974) found that type 1 pilated gonococci showed greater attachment to human spermatozoa than non-pilated type 4. Attachment of either type was unaffected by heat or formaldehyde which suggests that viability was not a factor. It was also shown that incubation of type 1 organisms with antiserum to pili reduced attachment to levels observed for untreated type 4, although the purity of the pilus preparation used to induce antibody was questionable. In addition to these findings, the authors also observed that incubation of other type 1 and type 4 organisms with antiserum did not affect attachment. Unfortunately, no attempt was made to separate the unattached bacteria from spermatozoa before counting and this could lead to unreliable data.

Punsalang and Sawyer (1973) claimed that only types 1 and 2 gonococci attached to buccal epithelial cells, agglutinated erythrocytes from various mammalian sources and resisted phagocytosis by rabbit or human leucocytes, whereas type 4 gonococci did not. Trypsinization or pre-treatment of gonococci with antibody to pili inhibited both hemagglutination and attachment to buccal cells in vitro and rendered type 1 cells more suseptible to phagocytosis. It was also found that rabbit erythrocytes attached to type 1 but not to type 4 colonies. None of the carbohydrates tested including D-glucose, maltose, D-mannitol, dulcitol, D-sorbitol, raffinose, saccharose, lactose, D-fructose, D-galactose, D-mannose and inulin, nor treatment of erythrocytes with trypsin or neuraminidase inhibited hemagglut-

ination by type 1 organisms.

A fallopian tube model has been used by Ward et al. (1974) who showed, with electron microscopy, microvilli of the fallopian tube mucosa adherent to pilated gonococci with subsequent penetration of the mucosal lining and establishment of infection in the lamina propria. Although large numbers of gonococci were attached to secretory cells the ciliated cells were essentially free of adhering and intracellular gonococci.

Swanson et al. (1974) extended earlier work with the use of ^{14}C- labelled gonococci and found that the type 2 cells remained associated to a greater degree with Hela cell monolayers than did the type 4 cells. Swanson et al. (1975) also labelled gonococci with ^{125}I and observed that type 2 showed a greater degree of association with HAM, foreskin, and Hela cells in contrast with type 4. Trypsin treatment of the gonococci, in contrast to the findings of Punsalang and Sawyer (1973), did not alter the level of attachment of any of the gonococcal types with tissue culture cells. Swanson et al. (1974) had found that trypsinization of colony type 2 cells, apparently, did not effectively depilate the gonococcal cells nor visibly alter the morphology of their pili, leaving attachment via pili still in question. The ineffectiveness of trypsin treatment on attachment of the gonococcus suggests that aside from pili other trypsin resistant proteins or non-proteinaceous adhesins may be involved in attachment.

Tebbutt et al., (1976) showed that both pilate and non-pilate strains attached to all human and guinea pig tissues employed. The human tissues were sections from adult endocervix, fallopian tube epithelium, ectocervix and bronchial epithelium. Guinea-pig

tissue sections were taken from the male posterior urethra, bladder and the female uterine horn and cervix. Adherence was assessed by determining the number of gonococcus colony-forming units (CFU) in the vigorous and gentle washings and in the tissue homogenate. The results were expressed as percentage of viable organisms remaining with each fraction. The pilated strain, similar to earlier reports, adhered significantly better than the non-pilated strain to human endocervix, ectocervix and fallopian tube sections, but did not adhere significantly better to human bronchus or any of the guinea-pig tissue sections. Since the non-pilated strain did adhere a non-pilar adhesin is suggested.

Mardh and Westrom (1976) used human vaginal cells and a visual counting system (mean number of gonococci per cell) to show that freshly isolated type 1 gonococci adhered better to these epithelial cells than two type 4 laboratory strains.

Tramont (1976) has demonstrated inhibition of attachment of a number of gonococcal strains to buccal mucosal cells with rabbit antigonococcal antisera. The antisera were absorbed with non-pilated organisms of the same strain to render them specific for pili. It was found that the antisera inhibited the attachment of the homologous organism at the highest titer in nearly every case, although higher antibody concentrations inhibited adherence of different strains as well. Pre-immunization antisera, or antisera raised to non-pilated gonococci did not inhibit adherence.

Ashton et al. (1977) isolated an IgG-containing fraction from goat's milk before and after instillation of the mammary gland with N. gonorrhoeae, colony type 1 cells. It was found that

postinstillation IgG effectively reduced adherence of the homologous strain to Rhesus monkey kidney cells (RE2), derived from tissue culture. The adhering gonococci were visually counted by light microscopy and adherence was reported as the percentage of RE2 cells with one or more gonococci adhering. Preinstillation IgG gave little or no inhibition of adherence of seven heterologous strains. Absorption of the postinstillation IgG with either whole gonococcal cells or rabbit antigoat IgG removed its ability to inhibit adherence. Three strains of gonococci of types 1 and 4 were tested for adherence and in every instance the type 1 cells adhered significantly better than type 4 cells.

Ward and Watt (1977) have indicated that when fallopian tube organ cultures were challenged with gonococci coated with anti-pilus antibodies, organisms were still seen adhering closely to the epithelial cells with some penetration occurring. However, antiserum against lithium acetate-extracted outer membranes of the P9 strain prevented its adherence and subsequent penetration of the fallopian epithelium. This suggests that pili are not absolutely required for adherence, but that other surface components are involved.

Tramont and Wilson (1977) have provided evidence, supporting the findings of others, that there is a strain-to-strain variation in the attachment ability of gonococci. They used human buccal cells and assessed adherence by visually counting the adhering gonococci. They found that the buccal cells varied in their day-to-day capacity to support adherence of several gonococcal strains. Strains of gonococci subcultured daily <u>in vitro</u> also differed in their day-to-day ability to adhere to the same buccal cells kept

over a period of twenty-two days. When several strains of gonococci were tested with buccal cells from different donors the percentage of buccal cells with adhering gonococci varied from donor to donor with each strain. The range was 0 to 60 %.

Surface charge has been suggested as a factor which might affect bacterial-host interactions (Jones, 1977; Heckels et al., 1976). Heckels et al. (1976) used isoelectric focusing to show that the gonococcus has a net negative surface charge. They chemically modified the surface amino or carboxyl groups, which changed the charge on the gonococcus and thereby altered the ability of the organism to adhere to HAM cells from tissue culture. Pilated gonococci attached better than non-pilated gonococci when a net negative (unmodified) charge was present, but did not attach better when the carboxyl and amino groups were blocked by formaldehyde, methylamine hydrochloride, 1-ethyl-3(dimethylaminopropal) carbodiimide and $MgCl_2.H_2O$. Adherence of types 1 and 4 was increased as the negative charge was reduced and replaced by a more positive charge. It was suggested that pili may help to overcome the apparent long range electrostatic repulsion, thus giving pilated cells an advantage in attachment over the non-pilated types. Since eucaryotic cells carry a net negative charge as well (Jones, 1977) it would appear that long range electrostatic repulsion may decrease the approach and attachment of these cell types.

Pearce and Buchanan (1978) studied optimum conditions of adherence of ^{125}I-labelled gonococcal pili to various human cells. These authors were able to show that buccal and cervical-vaginal cells, fallopian tube mucosa and sperm bound a greater number of pili per unit area than did fetal tonsil fibroblasts, Hela M cells,

erythrocytes and polymorphonuclear leucocytes. Although pili are antigenically heterogeneous it was found that antigenically distinct pili from four strains of gonococci attached equally well to a given cell type. Attachment of pili reached saturation within an hour and was temperature and pH dependent, with optimum attachment occurring at 37^{o} C and a pH of about 4.5. Inhibition of pilus attachment by antipilus antibody did occur and was greatest when the homologous antibody was used which supports the previous findings of other investigators. Attachment of pili was enhanced in the presence of calcium, magnesium and iron, whereas it was decreased by heat or ultraviolet light irradiation.

Buchanan et al. (1978) have demonstrated the inhibition of pilus attachment to various cells by gangliosides and heparin. Ganglioside is present in membranes of eucaryotic cells.

James et al. (1976) have extended their work to include chemical factors affecting gonococcal adherence to spermatozoa. Calcium and iron reduced and enhanced adherence respectively. ATP and cAMP decreased adherence. Trypsin, alpha-chymotrypsin and lysozyme all reduced adherence of type 1 cells, whereas neuraminidase had no effect.

Gubish et al. (1979) have used ^{32}P-labelled gonococci to show adherence to Hela cells. It was found that strain F62 type 2 cells attached better than the type 4 cells. Adherence varied from 0 to 22.5 gonococci per Hela cell, depending on the incubation conditions and the multiplicity of infection used. Trypsinization of the Hela cells decreased the adherence of both gonococcal types. However, adherence was restored to near-normal levels 24 hours after trypsin treatment, suggesting

regeneration of the surface component involved in adherence.

Methods of Assaying Bacterial Adherence

A number of techniques have been used to detect adhering bacteria. These generally include the use of radioisotope-labelled bacteria and visual and viable counting (James-Holmquest et al., 1974; Swanson et al., 1975; Tebbutt et al., 1976; Salit and Gotschlich, 1977; Bartelt and Duncan, 1978; Johanson et al., 1979). These techniques can be used in various situations depending on the organism and system being studied. However, difficulties can arise with each of them.

The visual counting method usually involves the staining of the cells, and the associated bacteria are then counted by light microscopy. The result can be expressed as the mean number of bacteria per eucaryotic cell, but counting of more than 50 eucaryotic cells can be tedious if not impossible if large numbers of bacteria are adhering. This method is therefore restricted to assessing a relatively small population of eucaryotic cells.

The viable counting method depends solely on the viability of the bacteria. Adherence can be assessed by determining the number of viable units remaining with homogenates of eucaryotic cells or tissues. Alternatively the number of viable units removed from the cells or tissues by washing can be determined. The alternative method may be useful when large numbers of adhering indigenous flora exist or when homogenates cannot be made without killing the bacteria. A large number of eucaryotic cells can be assessed by this method.

Radiolabelled bacteria can be mixed with eucaryotic cells and then the unattached bacteria are removed after which the amount of

radioactivity remaining with the cells is determined. This gives the number of bacteria adhering where the specific activity of the bacteria is known. This method is objective and allows a large population of eucaryotic cells to be assessed. One problem in this method could arise in the radiolabelling of the bacteria.

CHAPTER III

MATERIALS AND METHODS

Physical and Chemical Methods

Suspending solutions: Modified Hank's balanced salt solution (MHBSS) and proteose peptone saline (PPS) were the two suspending solutions used in this study. Hank's balanced salt solution was modified by removal of magnesium, calcium and glucose and by the addition of ethylenediaminetetraacetic acid (EDTA) to 0.05%. This modification reduces the clumping of the buccal epithelial cells. The PPS described by Norrod and Williams (1979) contained 1% Proteose Peptone no. 3 (Difco) and 0.85% NaCl in double-distilled water with an unadjusted pH of 7.0 $\pm$ 0.2. The PPS was sterilized by autoclaving for 15 min. at 15 lbs/in^2 and 121^{o}C.

Spectrophotometry: Absorbance of epithelial and gonococcal cells was measured with a Unicam SP800B double-beam recording spectrophotometer (Unicam Instruments Ltd., Cambridge, England) set at 650 nm.

Nitrogen measurements: All gonococcus nitrogen determinations in this study were calculated on the basis of a graph which gave absorbance vs. nitrogen content.

Total cell counts: Counts of epithelial and gonococcal cells were obtained from a graph of absorbance vs. number of cells.

Measurement of CO_2: CO_2 tensions were measured with a Kwik-Chek apparatus (Burrell Corp., Pittsburgh, PA.).

Bacteriological Methods

Strains and identification: Seven strains were used in this

study. Strain F62, which had been previously subcultured in our laboratory, was originally obtained from the Centre for Disease Control (CDC) in Atlanta, Georgia (Chan, 1974). Strain 191, the reference strain, and strains 195,122, 633, 24479, and 44269 were isolates obtained from the Health Sciences Centre (HSC) bacteriology laboratory in Winnipeg. These strains had been previously subcultured a number of times in our laboratory on artificial media and were stored frozen. All strains were tested for fermentation of glucose, sucrose, maltose and lactose, for oxidase and were Gram-stained to confirm that they were N. gonorrhoeae. Gonococci utilize glucose only.

Colony types: Colonial types 1 and 4 were used except in the case of strain F62 where colony type 2 was used. Colonial morphology determinations were based on characteristics described in Table I. Colonies were viewed with a Bausch and Lomb stereomicroscope (Model 7) with diffuse substage and oblique above stage lighting. Electron microscopic examination of cells from type 1 and type 2 colonies showed the presence of pili. No pili could be seen on cells from type 4 colonies.

Maintenance and preservation: Both colonial types of each strain were maintained on a solid medium, GCBD, containing per liter: 36 g. of GC agar base (BBL); 20 ml of supplement (See Appendix A). Several colonies of the desired type were selected and transferred daily onto the above medium. Inoculated GCBD plates were incubated for 18-24 hours in a humid Hotpack CO_2 incubator (Hotpack Canada Ltd., Waterloo, Ontario) set at 36^{o}C with an atmospheric CO_2 tension of 3-5 %.

Strains were preserved by the method of Ward and Watt (1971). Heavy suspensions of gonococci, grown on GCBD, were

made in a solution containing 8% glycerol and 1% proteose peptone (Difco) in double-distilled water. Aliquots of this suspension were placed in sealed glass ampoules which were stored in a liquid nitrogen cryostat. Some aliquots were also placed in one dram vials which were then stored in a freezer unit set at -70°C.

Viable counts: All viable counts were performed by a modification of the method of Miles et al. (1938) based on the recent work of Slack and Wheldon (1978). Instead of dropper pipettes, an Oxford micropipette (Oxford Laboratories) delivering a fixed 25 µl volume and fitted with a standard disposable plastic tip was used to place drops on pre-dried GCBD plates. Ten-fold and 4-fold dilutions were made in PPS and ten drops of the appropriate dilutions were placed on an agar plate. Only drops containing 30-300 colonies were counted. The percentage of different colonial types in each drop was assessed as well.

Viability of gonococci in PPS and MHBSS: An experiment was conducted in which the viability of gonococci was determined in PPS and MHBSS suspending fluids. The procedure was as follows: Gonococci were removed from GCBD plates after 22 hours of growth, suspended in PPS and MHBSS and adjusted to 10^8 ml $^{-1}$ (total count). The suspensions were allowed to stand at 20 ± 2°C and samples for viable count determinations were taken at 0 time and then at 30 min. intervals up to and including 180 min.

Culturing in liquid medium: The ESP liquid medium used throughout this study was that described by Chan et al. (1975). It contains, per liter of double-distilled water: Proteose Peptone no. 3 (Difco), 15 g.; dipotassium phosphate, 4 g.; monopotassium phosphate, 1 g.; NaCl, 5 g.; and supplement, as

in appendix A, 20 ml. Erlenmeyer flasks (250 ml) containing 25 ml of ESP were inoculated to a final concentration of approximately 10^6-10^7 colony-forming units (CFU) ml^{-1}. The inoculum was prepared by suspending gonococci of the appropriate colony type, which had been growing on GCBD for about 24 hours, in ESP. The inoculated flasks were placed in an Incubator Shaker, model R-25, (New Brunswick Scientific Co., Inc., New Brunswick, New Jersey) set at a shaking rate of 100-120 rpm at a temperature of 37°C. An above flask atmosphere at 16% CO_2 tension was established and maintained by introducing into the incubator pre-mixed compressed air and CO_2 as established by the flow ratio between twin flow meters (Matheson of Canada Ltd., Whitby, Ontario).

Radiolabelling of gonococci: Gonococci, strain 191, types 1 and 4 were radiolabelled indirectly by growth in ESP. The ESP contained low or high specific activity D-(U-^{14}C) glucose or (U-^{14}C) algal protein hydrolysate (New England Nuclear, Boston, Mass., and Amersham Corp., Des Plaines, Ill.). Radioactive concentrations were 0.5, 0.8, and 1.0 μCi ml^{-1} respectively. In all experiments a 1.0 ml aliquot was taken from the flask cultures at 0 time and every 4 hours thereafter up to and including 24 hours of incubation. Typically, each sample was then mixed (Vortex Junior Mixer) for two min. and the absorbance was measured. The aliquot was centrifuged at 4°C in a Lourdes 10R-Clini-Fuge (Lourdes Instrument Corp., Old Bethpage, New York) at 3000 x g for 20 min. The resulting pellet was resuspended in MHBSS and washed four more times (3 ml each wash) by centrifugation. The final pellet was dried for 18-24 hours at 37°C or until completely dry. The pellet was then solubilized with 0.5 ml formamide

(Fisher Scientific) and heated for approximately 3 hours at 60°C. Aliquots (50 µl) of this solution were placed in scintillation vials with 10 ml of scintillation fluid containing per liter of scintillation grade toluene (Fisher Scientific): PPO, 3 g.; POPOP, 0.1 g.. Each sample was counted for 10 min. in an LS-100 Liquid Scintillation System beta-spectrometer (Beckman Instruments Inc., Irvine, California) with a preset window for ^{14}C. Specific activity was calculated as counts per minute (cpm) per µg of gonococcal nitrogen.

Gonococci were radiolabelled with ^{125}I by the method of Marchalonis et al. (1971) as modified by Swanson et al. (1975). Types 1 and 4 of strain 191 were grown on GCBD for 22 hours. The colonies of the desired type were then removed by adding several ml of PPS to the agar plates and scraping the surface with a bent glass rod. The thick gonococcus suspension was placed in fresh PPS with a Pasteur pipette and mixed for 2 min. to break up any gonococcal clumps. The absorbance of the suspensions was adjusted to 0.45 and 0.5 respectively to give a total count of 5×10^9 ml^{-1} for each colonial type. Twenty ml of each of the above suspensions was added to 50 ml polycarbonate Oak Ridge-type tubes and then centrifuged at 16,000 x g for 10 min. at 20 ± 2°C in a Servall RC-2 centrifuge (Ivan Sorvall Inc., Norwalk, Connecticut). The supernatants were poured off and the pellets resuspended in 1.0 ml of 0.01 M phosphate buffered saline (PBS) pH 7.5 and warmed to 30-35°C in a water bath. To each of these was added 100 µl of lactoperoxidase (0.8 mg ml^{-1}) purchased from Sigma, and then 25 µl (500 µCi) of carrier-free ^{125}I as $Na^{125}I$ (Amersham Corp., Des Plaines, Ill.). To start and maintain the iodination reaction, 100 µl of 0.01 M H_2O_2,

obtained as 30% H_2O_2 (Fisher Scientific Co.) was added, with thorough mixing at 0, 2.5, 5.0 and 7.5 min. throughout the 10 min. labelling period. The reaction was stopped by the addition of 30 ml of chilled PPS containing 5 mM cysteine and agitated on the vortex mixer. The suspensions were centrifuged at 16,000 x g for 10 min. as before. The resulting pellets were washed thrice with PPS without cysteine (30 ml each time) by centrifugation. The final pellets were resuspended in PPS and adjusted to 10^9 ml^{-1} (total count) with a Klett-Summerson colorimeter (Model 800-3, Klett Mfg., Co., New York) with a no. 42 filter. The Klett had been standardized against the Unicam SP800B spectrophotometer.

Specific activity of ^{125}I-labelled gonococci was assessed with 100 µl aliquots of the final suspension. Equal volumes of gonococcus-free filtrate, obtained by filtering 2-3 ml of the final suspensions through 0.22 membrane filters (Millipore Corp.) were also assessed for radioactivity. These aliquots were placed in 12 x 75 mm "snap-top" polystyrene tubes (Falcon, Oxnard, CA.) and counted for 1 min. in an Iso-Computron 1200 gamma-spectrometer (General Medical Systems, Garland, Texas). Counts obtained for the gonococcus-free filtrate were subtracted from counts in the samples containing gonococci thereby giving cpm per 10^8 gonococci which was converted to cpm per µg N.

Epithelial Cells

Preparation and viability: Human buccal mucosal (squamous) epithelial cells (BMEC) were scraped from the epithelium of the lips and cheeks of the author with a wooden tongue depressor and were washed off with a Pasteur pipette into about 20 ml of either MHBSS or PPS. The cells were then sedimented and washed by

centrifugation at 20 ± 2°C for 6 min. at 300 x g in a Lourdes centrifuge. The supernatant was pipetted off and the cell pellet was washed thrice (5 ml each wash). Cells from the pellet in the final wash were resuspended and adjusted to the appropriate concentration. Gram-stained buccal cells were examined with a light microscope (Wild Leitz Can., Ltd., Ottawa, Ont.) and very few indigenous bacteria could be seen adhering to the cells. Usually a mean of less than one bacterium per cell was obtained. The diameter of the BMEC ranged from about 30-50 μ. Their viability was tested with trypan blue (King et al., 1959) in both MHBSS and PPS immediately after harvesting without the normal washing procedure. A 0.5% aqueous solution of trypan blue was prepared and 0.2 ml of this was added to 1.8 ml of the epithelial cell suspension. A Neubauer counting chamber was filled with this suspension and the cells were viewed at a magnification of 500 x with a light microscope. The mean viability of the cells in six experiments was 1.5 ± 0.24 %. There was no significant difference in the viability of cells suspended in MHBSS or PPS.

Adherence Methods

Viable count assay: Gonococci were grown for 22 hours on GCBD as described previously. Isolated colonies of the desired type were transferred via loop into PPS warmed to 37°C and the suspension vortexed for 30-60 sec. The gonococcus concentration was adjusted to 10^9 ml^{-1} (total count). Buccal cells prepared in PPS were adjusted to approximately 10^6 per 0.9 ml. To a 10 x 75 mm glass test tube (Pyrex no. 9820) 0.9 of BMEC suspension was added, followed by 0.1 ml of gonococcus suspension to give a gonococci:BMEC ratio of 100:1. A control included gonococci plus

0.9 ml of PPS. The tubes were sealed with parafilm and shaken at 20 ± 2°C for 60 min. with a Burrell "Wristaction" shaker (Burrell Corp., Pittsburgh, PA.) set for maximum angle of swing. All tubes were then centrifuged at 60 x g for 5 min. in a Lourdes centrifuge at 20 ± 2°C to sediment the BMEC. An aliquot of the supernatants was carefully removed without disturbing the epithelial cell pellet, after which the gonococcus CFU ml^{-1} was determined. Percent adherence was calculated as follows:

$$\left|\frac{\text{CFU in BMEC-free supernatant}}{\text{CFU in control supernatant}} \times 100 - 100\right| = \% \text{ Adherence}$$

No colonies of indigenous flora were observed when the gonococcus colonies were being counted and therefore did not interfere with the viable counts. The absence of such colonies is likely due to the removal of most of the nonadhering bacteria with the thorough washing of the buccal cells when they were being prepared. The nonadhering indigenous flora not removed at this stage were probably eliminated when dilutions of the supernatants to determine gonococcus CFU were being made.

Visual count assay: Gonococci were grown in ESP medium as described previously. Samples were removed near the end of the exponential growth phase (about 12 hours) and washed as in the ^{14}C labelling procedure. Buccal cells were prepared in MHBSS. Gonococci and BMEC were adjusted to a ratio of 150:1 and shaken in universal bottles for 15 min. at 20 ± 2°C with a Burrell shaker. Earlier experiments had shown that this ratio was near that which saturated the receptor sites on the BMEC. Unattached bacteria were separated from the BMEC by seven washings (3 ml MHBSS each wash) by differential centrifugation with a Lourdes centrifuge at 300 x g for 6 min. at 20 ± 2°C. The buccal cell pellet in the final wash was then spread onto standard glass slides,

air-dried, heat-fixed and Gram-stained. The number of Gram-positive and negative bacteria was counted on fifty buccal cells. Buccal cells without gonococci added served as the control.

Radioisotope count assay: Gonococci labelled with ^{14}C were prepared and shaken with BMEC in the same manner as for the visual counting procedure. Typically, aliquots of the gonococcus-BMEC suspension were then passed through 25 mm 12 μ pore size nitrocellulose filters (Schleicher and Schuell, Keene, N.H.) with a Millipore filtration apparatus connected to a vacuum pump (model 13152, Gelman Instrument Co.). Approximately 10 ml of MHBSS was then passed through each filter. The filters were placed in scintillation vials and 0.5 ml of formamide was added to each vial. The vials were capped and allowed to stand overnight at 37°C. Scintillation fluid (10 ml) was added to each vial and the samples were counted as in the ^{14}C-labelling procedure. Controls included filtration of equal numbers of gonococci without BMEC through the same filters and also through 0.22 μ filters. Percent adherence was calculated as follows:

$$\frac{\text{cpm remaining with BMEC}}{\text{total cpm added}} \times 100 = \% \text{ Adherence}$$

Gonococci labelled with ^{125}I were mixed with BMEC in the same manner as described in the viable count assay. Non-adherent gonococci were removed by filtration as described above. Briefly, 0.5 ml aliquots of the gonococcus-BMEC suspension were filtered and 10 ml of PPS was passed through each filter. The filters were rolled onto short pieces of glass tubing wrapped with masking tape (sticky side out) and placed in 12 x 75 mm polystyrene tubes and counted as described in the ^{125}I labelling section. Controls were the same as those with the ^{14}C-labelled gonococci with an

additional one. This included pre-filtering buccal cells onto 12 μ filters and then passing gonococci alone through the BMEC-laden filter. Percent adherence was calculated as above.

Inhibition of adherence with carbohydrates and lectins: Carbohydrates and lectins were dissolved in either PPS or saline (0.85%) to make concentrated solutions. Twenty-five μl of these solutions were added to 10^6 BMEC (total count) suspended in 0.9 ml PPS after which 10^8 gonococci (total count) were added. The viable count assay procedure was followed in testing the inhibitory effects of these compounds.

Carbohydrates were obtained from Applied Science Laboratories Ltd., Sigma Chemical Co., and Aldrich Chemical Co., Inc.. Lectins were obtained from Sigma Chemical Co..

Statistics

The percentage of adherence was calculated with analysis of variance and the two-sample T-test. Linear regression analysis was used to compare percent adherence with percent viable gonococci in inoculum. These tests were performed on a computer as programmed by the Health Sciences Computer Department of this University.

CHAPTER IV

RESULTS

The first part of the present study concerns the growth of gonococci in liquid medium to be used in radiolabelling the organism and investigates the maintenance of viability of gonococci in suspending solutions to be used where viability is essential. Several methods of assaying adherence are also examined with one selected to investigate factors affecting gonococcal adherence.

A. Growth and Viability

Growth of strain 191 types 1 and 4 N. gonorrhoeae in ESP liquid medium: Gonococci were grown in ESP liquid medium, as described elsewhere in "Material and Methods". Growth was assessed as a change in absorbance rather than change in CFU ml^{-1}. Both types 1 and 4 showed good growth over a 24 hour incubation period (Figures 2 and 3), with growth of type 4 being slightly better than that of type 1. However, a relatively large inoculum of approximately 10^6-10^7 CFU ml^{-1} was required for both types. A lag phase of approximately 4 hours was observed for both types followed by a relatively rapid exponential phase of growth ending about 12 hours from inoculation. After this a decrease in the absorbance was noted. This decrease is probably due to the autolysis of the gonococcus in unfavorable environmental conditions. A 100-fold increase in gonococci total count was observed for both T1 and T4 colonial forms.

Viability of strain 191 types 1 and 4 N. gonorrhoeae in PPS and MHBSS: The survival of gonococci in PPS and in the less complex MHBSS was investigated. Viability of types 1 and 4 was maintained in PPS for 180 min. (Figure 4). Type 1 gonococci suspended in MHBSS showed a rapid decrease in viability over the same time. PPS was selected

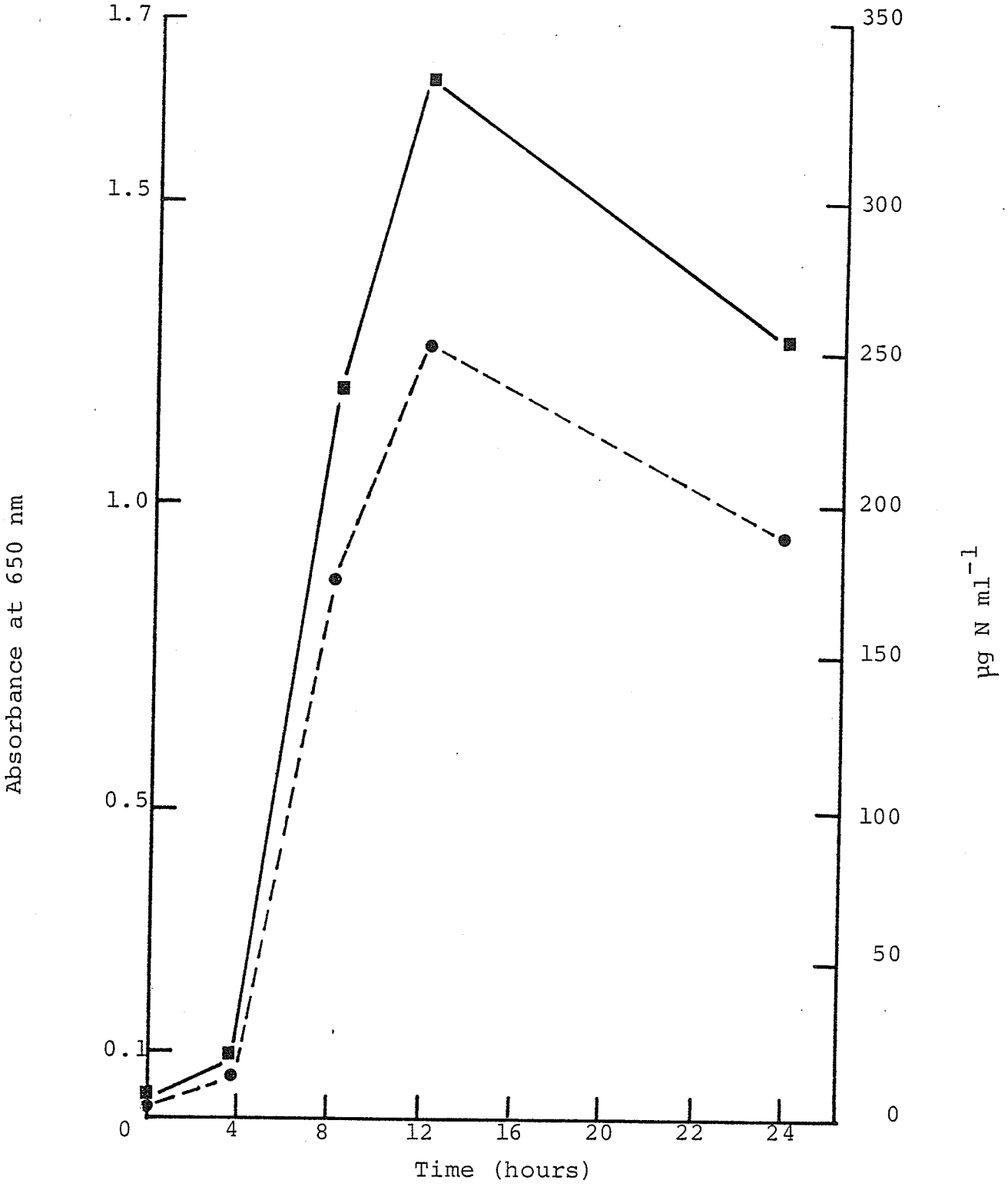

Figure 2. Growth of strain 191 type 4 N. gonorrhoeae in enriched single phase liquid medium.

■—■ represents absorbance.

●--● represents µg N ml^{-1}.

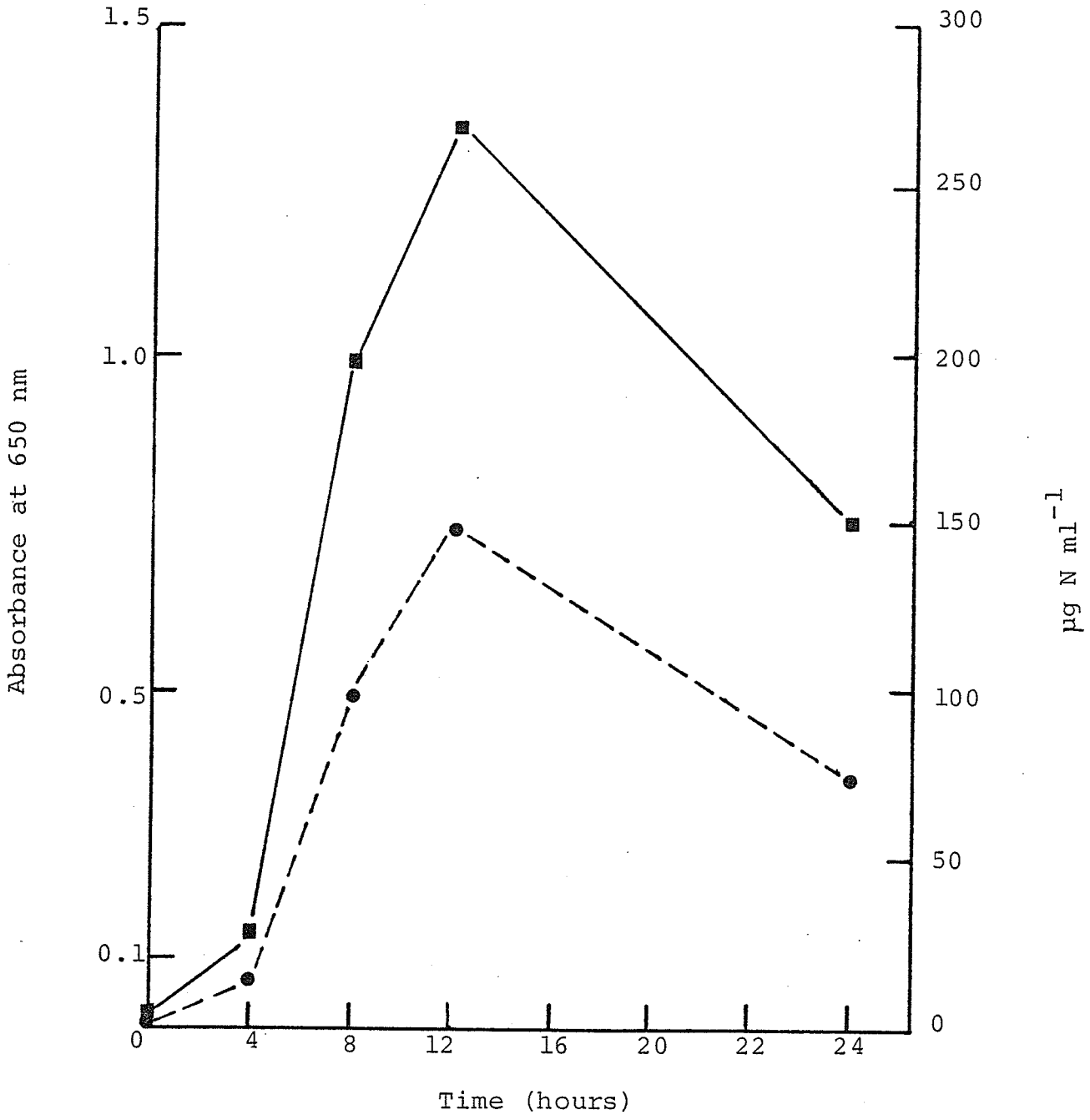

Figure 3. Growth of strain 191 type 1 N. gonorrhoeae in enriched single phase liquid medium.

■—■ represents absorbance.

●--● represents µg N ml^{-1}.

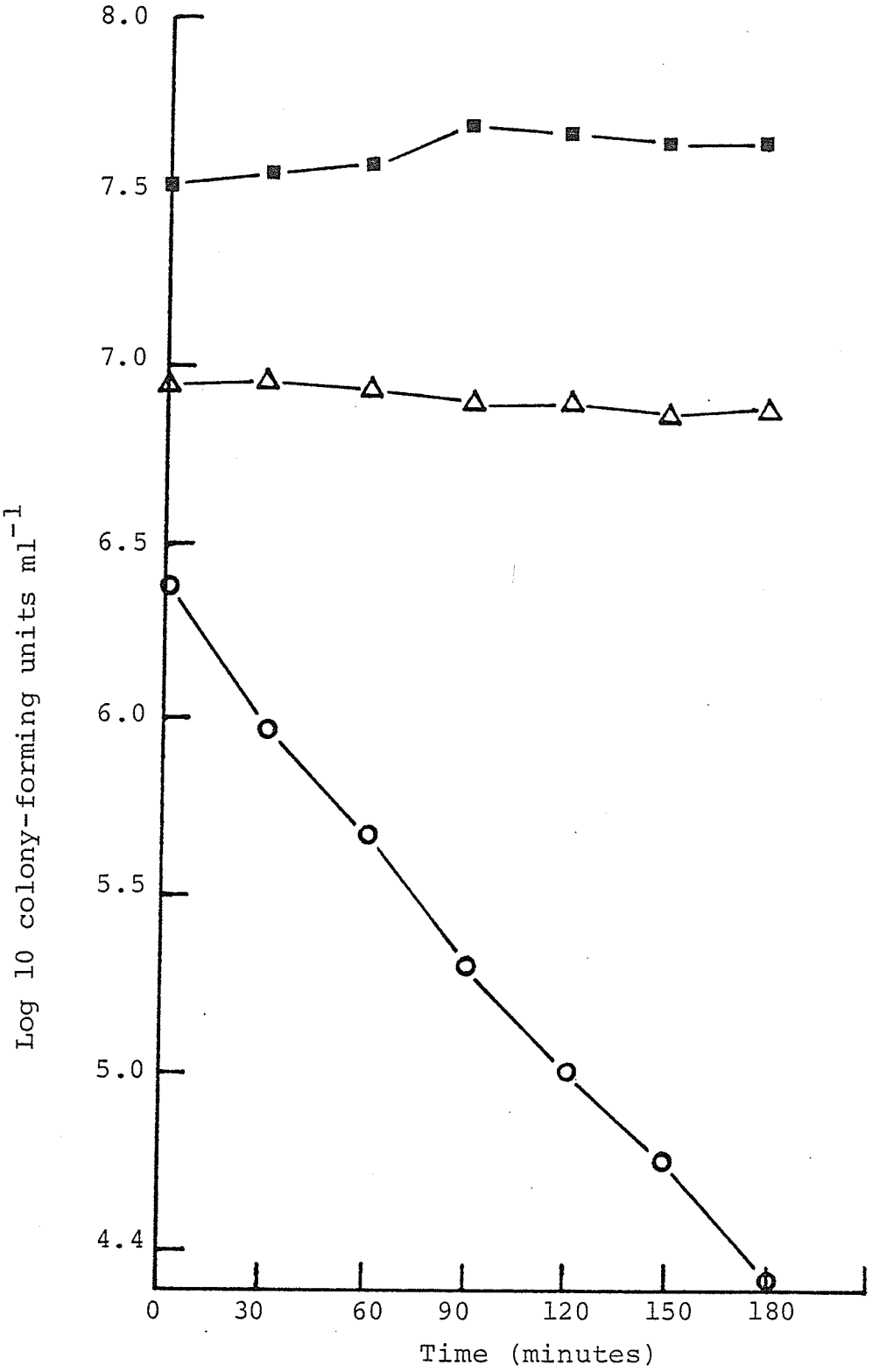

Figure 4. Survival of strain 191 types 1 and 4 N. gonorrhoeae in proteose peptone saline solution and modified Hank's balanced salt solution (20 ± 2°C).

■—■ type 1 in proteose peptone saline solution.
△—△ type 4 in proteose peptone saline solution.
○—○ type 1 in modified Hank's balanced salt solution.

as the standard suspending solution when the viable count adherence assay was being used, provided that the 3 hour period was not exceeded in the experiment.

B. Methods of Assessing Adherence

Indirect radiolabelling of strain 191 types 1 and 4 N. gonorrhoeae with ^{14}C: In a number of adherence studies radiolabelling of gonococci has been used as a method of assessing the numbers of gonococci adhering to various eucaryotic cells. In this study, radiolabelling of gonococci with ^{14}C and ^{125}I was undertaken for the same purpose. Initial labelling of strain 191 types 1 and 4 was achieved with low specific activity uniformly labelled ^{14}C glucose (D-(U-^{14}C) glucose). This substrate was selected on the basis that all gonococcal strains utilize it as an energy source. Over the 24 hour labelling period both gonococcal types took up the ^{14}C label with maximum activity in the case of type 1 being reached at about 12 hours (Table II). Type 4 gonococci labelled twice as well as type 1 cells, which probably reflects increased ability of the former to better utilize the glucose. The activities obtained at 12 hours using low specific activity glucose at a radioactive concentration of 0.5 $\mu Ci\ ml^{-1}$ were very low, being 147 cpm μg^{-1} gonococcal N for type 1 and 311 for type 4. These activities represented 10^8 gonococci (total count).

Since activities obtained with low specific activity glucose were small, an attempt was made to increase the level of labelling with ^{14}C glucose of a greater specific activity while also varying the unlabelled glucose concentration in the ESP medium to reduce the competition between labelled and unlabelled glucose. Type 4 cells were used in this experiment since the relative radiolabelling trend of both types was known from the previous

TABLE II

RADIOLABELLING OF N. GONORRHOEAE, STRAIN 191, TYPES 1 AND 4 WITH D-(U-^{14}C) GLUCOSE OF LOW SPECIFIC ACTIVITY [a]

Hours of growth	Type 1		Type 4	
	µg N ml^{-1} [b]	Specific activity [c]	µg N ml^{-1}	Specific activity
0	1.1	0	2.6	0
4	8.7	19	8.9	43
8	80	81	125	89
12	140	147	179	311
24	120	77	133	317

a Specific activity of the D-(U-^{14}C)glucose is 4.346 mCi mmol^{-1} as purchased, but the final radioactive concentration in the medium was 0.5 µCi ml^{-1}.

b µg gonococcal N ml^{-1} culture medium.

c cpm ^{14}C µg^{-1} gonococcal nitrogen.

experiment. The unlabelled glucose concentration was reduced from 0.2% to 0.025% in two-fold dilutions. A decrease in growth was observed as the concentration of unlabelled glucose decreased (Table III). Although a steady increase in the specific activity of the gonococci was observed at the end of 4 hours with a decreasing glucose concentration, this trend was not continued. However, the largest specific activity was found at the 8 hour sampling time in gonococci grown with 0.1% unlabelled glucose but this specific activity only represented a two-fold increase over that seen in type 4 cells labelled with the low specific activity glucose (Table II). A uniformly labelled ^{14}C algal protein hydrolysate was used as an alternative source of ^{14}C for labelling the gonococci as this hydrolysate contains a large cross section of the amino acids which gonococci may use. It was thought that the gonococci might incorporate more of the ^{14}C label than was the case with glucose. Here again as with glucose, the competing component in ESP, proteose peptone, was varied in concentration to reduce competition. Growth and specific activity were greater at 1.5% and 0.75% concentration of proteose peptone but were depressed by half at 0.375% (Table IV). Largest specific activities were obtained at the end of the lag phase (about 4 hours after inoculation) from gonococci grown in ESP with a proteose peptone concentration of 0.75%. Although the activity achieved under these labelling conditions was equivalent to that obtained with type 4 cells labelled with high specific activity glucose, the increase is only about four-fold over the activity obtained when these type 1 cells were labelled with low specific activity glucose. The radioactive concentration of the protein hydrolysate in the ESP medium was 1.0 $\mu Ci\ ml^{-1}$.

TABLE III

RADIOLABELLING OF N. GONORRHOEAE, STRAIN 191, TYPE 4 WITH D-(U-^{14}C) GLUCOSE OF HIGH SPECIFIC ACTIVITY [a]

	Unlabelled glucose concentration % (weight/volume)							
	0.2		0.1		0.05		0.025	
Hours of growth	µg N ml^{-1} [b]	SA[c]	µg N ml^{-1}	SA	µg N ml^{-1}	SA	µg N ml^{-1}	SA
0	3	0	3	0	3	0	3	0
4	45	17	29	47	18	73	28	97
8	264	410	240	663	186	435	162	466
12	267	407	251	563	192	435	177	381
24	147	ND[d]	128	507	115	ND	111	467

a Specific activity of the D-(U-^{14}C) glucose is 333 mCi mmol^{-1} as purchased, but the final radioactive concentration in the medium was 0.8 µCi ml^{-1}.

b µg gonococcal N ml^{-1} culture medium.

c Specific activity (cpm ^{14}C µg^{-1} gonococcal N).

d Not done.

TABLE IV

RADIOLABELLING OF N. GONORRHOEAE, STRAIN 191, TYPE 1 WITH (U-^{14}C) PROTEIN HYDROLYSATE [a]

Hours of growth	Proteose peptone concentration % (weight/volume) 1.5		0.75		0.375	
	µg N ml^{-1} [b]	SA [c]	µg N ml^{-1}	SA	µg N ml^{-1}	SA
0	2.1	0	2.1	0	2.1	0
4	15.2	581	17.3	668	8.9	431
8	135.5	64	118.7	255	69.3	182
12	157	122	133.4	209	73.5	263
24	72.5	141	46.2	144	36.8	317

a Specific activity of the (U-^{14}C) protein hydrolysate is 57 millicuries per milliatom of carbon as purchased, but the final radioactive concentration in the medium was 1.0 µCi ml^{-1}.

b µg gonococcal N ml^{-1} culture medium.

c Specific activity (cpm ^{14}C µg^{-1} gonococcal N).

Direct radiolabelling of strain 191 types 1 and 4 N. gonorrhoeae with ^{125}I: In the latter part of the adherence studies attempts were made to radiolabel gonococci with ^{125}I by the lactoperoxidase method. Table V shows the specific activities of types 1 and 4 labelled on two separate occasions. Activities reached by this method were sixty times that obtained in any of the indirect labelling experiments. A significant decrease in specific activities of both types occurred when the radiolabelling experiment was repeated, as shown in the Table.

Adherence of ^{14}C-labelled gonococci: Type 4 gonococci adhered better than type 1 with adherence of 59% and 43% respectively (Table VI).

Adherence of ^{125}I-labelled gonococci: The adherence seen in Table VI is similar to that for ^{14}C-labelled gonococci with type 4 gonococci adhering better than type 1. However the adherence of both types is lower than that observed with ^{14}C-labelled gonococci.

Adherence of visually counted gonococci: It was osberved with visual counting of gonococci on buccal cells that type 4 did not adhere in contrast with type 1 cells (Table VI). The adherence of type 1 cells was lower than with the other two methods of assessment. Few indigenous bacteria were seen on the prepared buccal cells (Figure 5). Buccal cells appeared to vary in their ability to adhere to gonococci. Counting of more than 50 buccal cells was tedious and therefore only a small number of cells could be assessed. Cells with large numbers of adhering gonococci could not be assessed accurately.

Adherence of gonococci assessed by viable counting: It was found by viable counting that type 4 gonococci did not adhere to

TABLE V

DIRECT RADIOLABELLING OF N. GONORRHOEAE, STRAIN 191, TYPES 1 AND 4 WITH ^{125}I BY THE LACTOPEROXIDASE METHOD [a]

Date labelled	Type	Specific activity [b]	cpm/ gonococcus
25-9-79	1	41,074	4.1×10^{-4}
27-9-79	1	23,067	2.3×10^{-4}
25-9-79	4	45,937	4.6×10^{-4}
27-9-79	4	15,921	1.6×10^{-4}

a Numbers of gonococci were the same in these experiments

b cpm ^{125}I μg^{-1} gonococcal N.

TABLE VI

ADHERENCE OF STRAIN 191 TYPES 1 AND 4 N. GONORRHOEAE TO BUCCAL CELLS AS DETERMINED BY THREE ASSAY METHODS

	% Adherence[a,b]			
Colony type	^{14}C	^{125}I	Visual count	Viable count
1	43	21	4	42
4	59	33	0	0

a For radiolabelled gonococci % Adherence =

$$\frac{\text{cpm on buccal cells}}{\text{total cpm added}} \times 100$$

For visual counting % adherence =

$$\frac{\bar{x} \text{ gonococci buccal cell}^{-1}}{\text{gonococci:buccal cell ratio}} \times 100$$

For viable counting % Adherence =

$$\left| \frac{\text{CFU in BMEC-free supernatant}}{\text{CFU in control supernatant}} \times 100 - 100 \right|$$

b Number of experiments performed for each assay method is as follows:

^{14}C - 2 experiments

^{125}I - 1 experiment

visual count - 2 experiments

viable count - 31 experiments for T1 gonococci

5 experiments for T4 gonococci

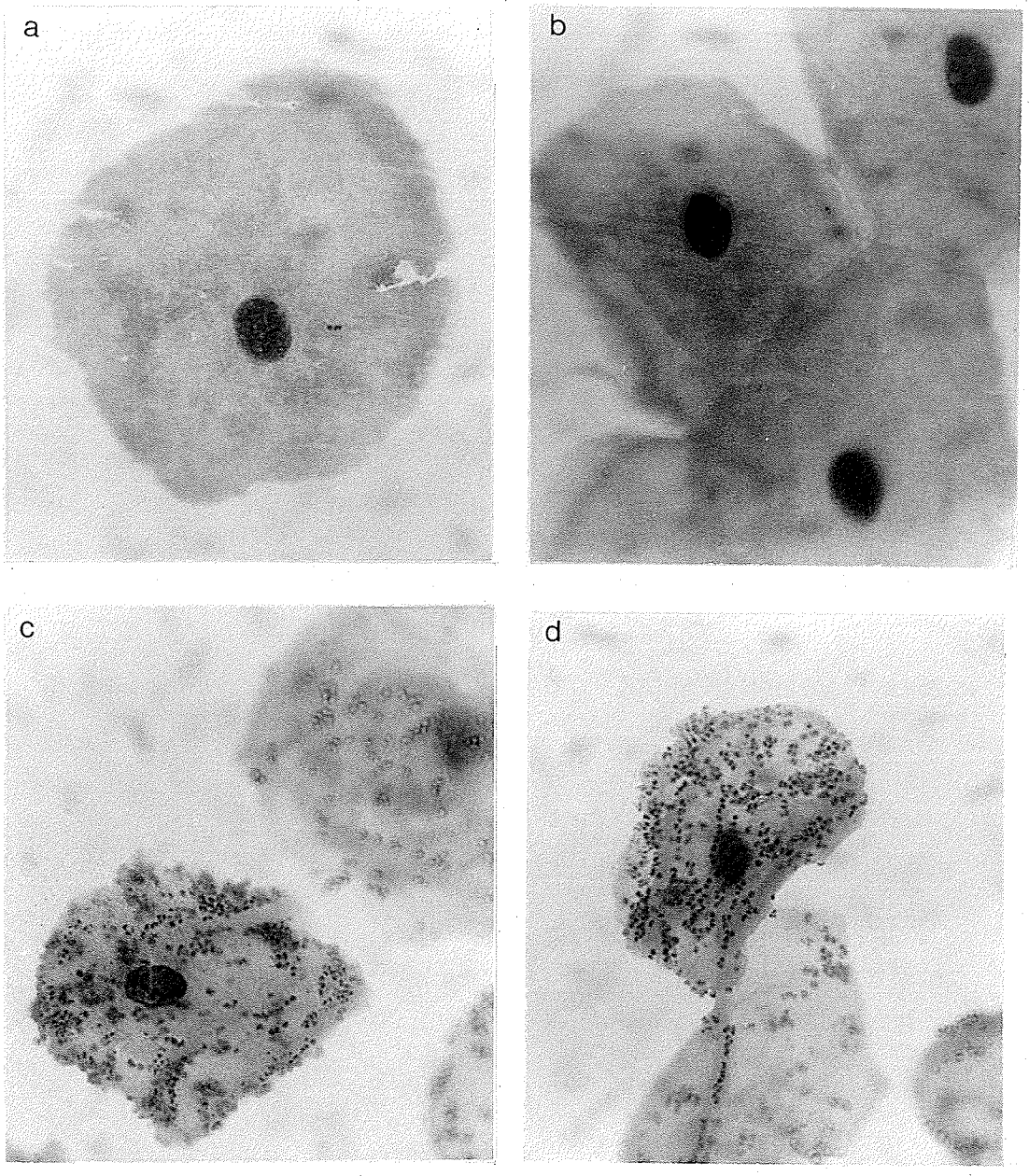

Figure 5. Gram-stained buccal cells. (x1200)

a,b. controls.
c,d. cells with adhering type 1 gonococci.

buccal cells whereas type 1 did. The adherence of type 1 cells as determined by this method was equal to or greater than that in the other two methods (Table VI). This method allowed large numbers of buccal cells to be assessed.

Summary of adherence assay methods: The visual and viable count methods were the only two which agreed with the literature. The results of these two methods indicate that type 4 gonococci did not adhere to buccal cells in contrast with type 1, which is the finding in the literature where buccal cells are used. Results found by the radiolabelling method showed adherence of type 4 to be greater than type 1, which is rarely observed in the literature. On the basis of these results the methods using radiolabelled gonococci were not selected for further work.

Visual counting was less favorable than viable counting since it was a more tedious method and few buccal cells could be assessed; thus the viable count method was selected for additional studies of gonococcal adherence.

C. Factors Affecting Adherence in Viable Count System

Effect of gonococci: buccal cell ratio on adherence: Gonococcal-buccal cell suspensions in this experiment were shaken for 15 minutes at 20 $\pm$ 2^{o}C. The percentage of the gonococcal inoculum adhering to the buccal cells showed a decline as inoculum size increased, except at a ratio of 500:1 where a large standard error exists (Table VII). The mean number of gonococci adhering to each buccal cell appears to increase to a maximum at a ratio of 500:1. A ratio of 100:1 was selected because its standard error was relatively small. Its use permitted fewer dilutions to be made of aliquots taken for CFU determinations, yet still eliminated any contaminating bacteria by dilution.

TABLE VII

EFFECT OF GONOCOCCI:BUCCAL CELL RATIO ON ADHERENCE [a]

Gonococci:buccal cell ratio	% Adherence ± S.E.[b]	Mean gonococci per[c] buccal cell
10	30 ± 6	3
100	14 ± 0.6	14
500	20 ± 9	100
1000	8 ± 8	80

a Gonococci and buccal cells were shaken for 15 minutes in proteose peptone saline solution at 20 ± 2°C.

b $\%\ \text{adherence} = \left| \frac{\text{CFU in BMEC-free supernatant}}{\text{CFU in control supernatant}} \times 100 - 100 \right|$

c $\bar{X} = \frac{\text{no. of gonococci adhering}}{\text{total no. of buccal cells}}$

Effect of time and pH on adherence: The gonococci:buccal cell ratio of 100:1 was used in testing shaking periods of 15, 30 and 60 minutes. There was no significant difference in adherence at 15 and 30 minutes (Table VIII). However, an increase in adherence of 35% over the 15 and 30 minute shaking times did occur when a 60 minute shaking period was used. Shaking times beyond 60 minutes were not investigated. Since adherence was greatest at 60 minutes, this time period was adopted.

As shown in Table IX there is no significant difference in adherence in the pH range of 5.2-7.9. As expected there was a decrease in gonococcal viability as the pH decreased. A pH of 7.0 $\pm$ 0.2 was chosen as the standard pH as it was the unadjusted pH of the PPS. In this experiment the pH of the PPS was read before and after shaking to ensure that no change had occurred.

Relationship between viability and adherence: Data from thirty-one experiments with type 1 cells from strain 191 were used to determine if there was a relationship between the viability of gonococci in the inoculum and adherence. The same number of gonococci was used for each experiment and viability ranged from about 5-30%. All experiments were carried out at 20 $\pm$ 2^{o}C in PPS (pH 7.0) with a shaking time of 60 minutes and a gonococci:buccal cell ratio of 100:1. In this relatively narrow range of viability no relationship could be found between adherence and viability of gonococci in the inoculum. The slope of the regression line is not significantly different from 0 (Figure 6).

Adherence of different strains to buccal cells: Adherence conditions were the same as those used in the previous experiment. The adherence of seven strains of N. gonorrhoeae was tested as shown in Table X. Strain 191 was used as the reference strain

TABLE VIII

EFFECT OF TIME ON ADHERENCE OF *N. GONORRHOEAE*, STRAIN 191, TYPE 1 TO BUCCAL CELLS [a]

Incubation time (minutes)	% Adherence ± S.E.[b]	Statistical significance[c]
15	22 ± 2.6	N.S.
30	23 ± 1.8	N.S.
60	34 ± 2.2	$P < 0.01$

a Gonococci and buccal cells were mixed in a ratio of 100:1 in proteose peptone saline solution and shaken at 20 ± 2°C.

b $\%\ \text{Adherence} = \left| \frac{\text{CFU in BMEC-free supernatant}}{\text{CFU in control supernatant}} \times 100 - 100 \right|$

c Analysis of variance.

TABLE IX

EFFECT OF pH ON ADHERENCE OF STRAIN 191 T1 *N. GONORRHOEAE* TO BUCCAL CELLS

pH of suspending fluid[a]	% Adherence ± S.E.[b]	Statistical significance[c]	gonococcus CFU/ml[d]
5.2	46 ± 4.4	N.S.	7.2×10^6
6.1	50.5 ± 4.6		1.2×10^7
6.95	47.2 ± 3		1.4×10^7
7.9	55.5 ± 4.6		1.3×10^7

a The pH of the proteose peptone saline solution was measured before and after the experiments to ensure that no change had occurred.

b $\%\ \text{Adherence} = \left| \frac{\text{CFU in BMEC-free supernatant}}{\text{CFU in control supernatant}} \times 100 - 100 \right|$

c Analysis of variance.

d The initial gonococcal count (total) was about $10^8\ ml^{-1}$.

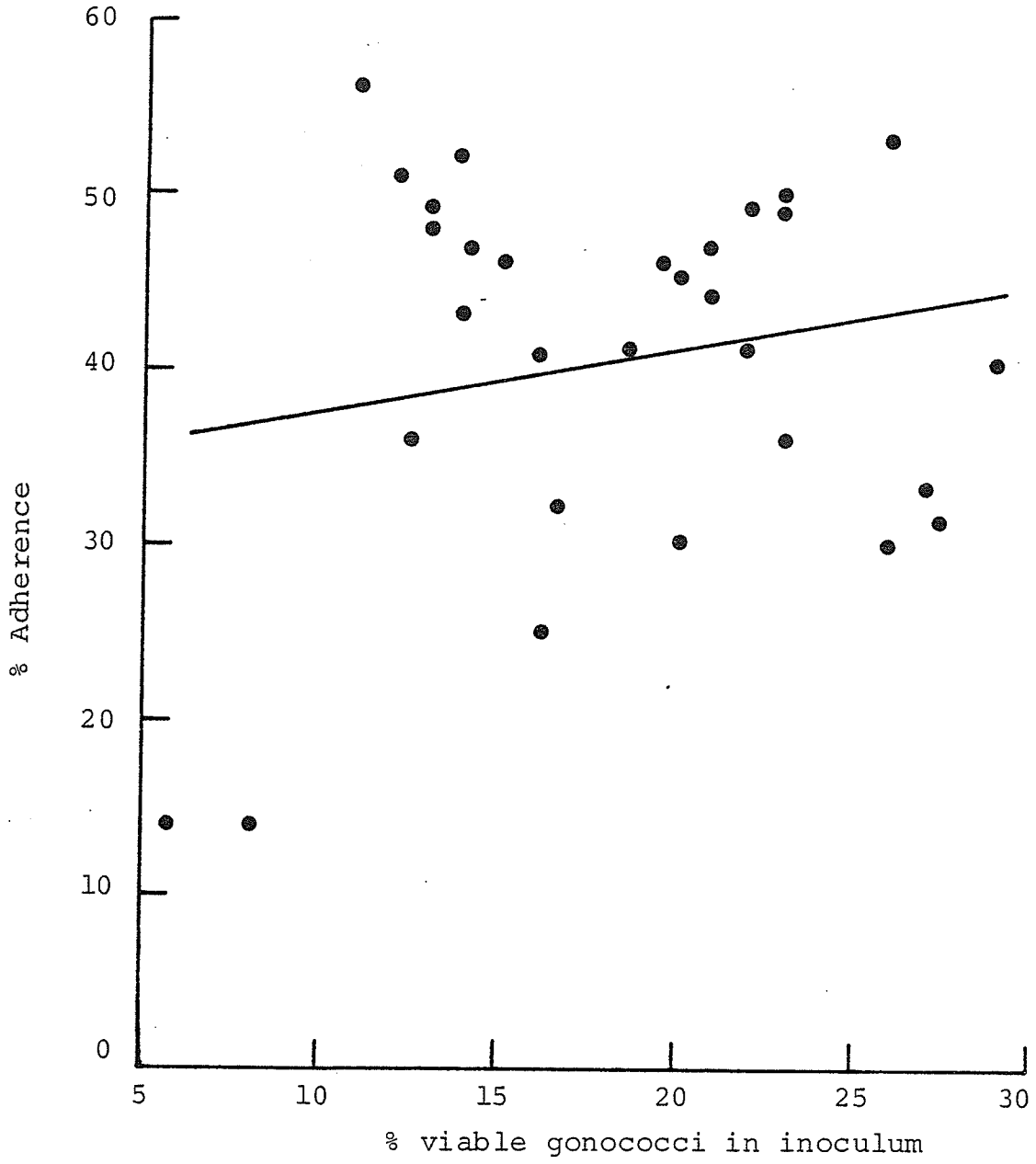

Figure 6. Relationship between gonococcal viability and adherence. Total number of gonococci added in each experiment was 10^8.

b = 0.265
r = 0.147
"t" Test H:b = 0
t = .789
P>0.4

TABLE X

COMPARISON OF ADHERENCE OF DIFFERENT STRAINS OF N. GONORRHOEAE TO BUCCAL CELLS USING STRAIN 191 T 1 AS A REFERENCE STRAIN

Strain	Colony type	% Adherence ± S.E.[a,d]	Adherence ratio[b,d]	% T4 CFU[c]
191	1	38.7 ± 3	1.0	1.3
195	1	55.1 ± 6.2	1.97	0
F62	2	66.1 ± 2.8	1.34	2.6
122	1	41.6 ± 2.7	1.19	2.3
633	1	27.9 ± 4.6	0.57	2.8
24479	1	19.5 ± 2	0.44	0.3
191,195, F62,122, 633,24479, 44269	4	0	0	100

a $\%\ \text{Adherence} = \left| \frac{\text{CFU in BMEC-free supernatant}}{\text{CFU in control supernatant}} \times 100 - 100 \right|$

b $\text{Adherence ratio} = \frac{\%\ \text{adherence of strain tested}}{\%\ \text{adherence of strain 191}}$

c Percentage of type 4 colonies was assessed in each experiment as colony counting was carried out.

d Data is based on one experiment per strain.

since its adherence characteristics were established in previous experiments. Cells of both types 1 and 4 colonies were tested, except for strain F62 where type 2 cells were used. Type 1 cells of strain 44269 were not used as they were unstable, yielding large numbers of type 4. The ratio for all type 4 cells was 0 in contrast to type 1 which ranged from 0.44-1.97. Each ratio was based on the adherence of strain 191 on a per experiment basis and not on the mean shown in Table X. For all type 1 populations of the 6 strains, only a small percentage of type 4 cells was observed.

Effect of carbohydrates, ganglioside, and lectins on adherence: Carbohydrates, ganglioside and lectins were added to buccal cells followed by the addition of the gonococci at final concentrations shown in Tables XI, XII, and XIII. Controls without carbohydrate, ganglioside or lectin were run parallel in each experiment. None of the carbohydrates at the concentrations tested inhibited adherence. Although D-galactose and alpha-methyl-D-galactoside caused some inhibition this was not reproducible even when a concentration of 1000 $\mu g\ ml^{-1}$ was used. Alpha-methyl-D-glucoside and glucose appeared to enhance adherence by 40-50%. In contrast, bovine ganglioside reproducibly inhibited adherence at a concentration of 1000 $\mu g\ ml^{-1}$. Inhibition of adherence by ganglioside at a concentration of 200 $\mu g\ ml^{-1}$ ranged from 0-30% and no inhibition was ever detected at 5 $\mu g\ ml^{-1}$.

Four lectins with known carbohydrate specificities were used in an attempt to block adherence. As shown in Table XIII none of the lectins inhibited adherence. However, wheat germ agglutinin, specific for N-acetyl-D-glucosamine, did decrease adherence initially, but this was not reproducible. Peanut and soybean agglutinins, specific for galactose and N-acetyl-D-galactosamine respectively,

TABLE XI

EFFECT OF CARBOHYDRATES ON ADHERENCE OF STRAIN 191 T1 *N. GONORRHOEAE* TO BUCCAL CELLS

Carbohydrate	Concentration $\mu g\ ml^{-1}$	Adherence ratio [a]	% inhibition
D-glucose	100	1.18	0
	5	1.4	0
D-mannose	100	0.97	0
	5	1.12	0
D-galactose	1000	0.99	0
	100	1.13	0
	5	0.78	0
	1	1.28	0
∝-Methyl-D-galactoside	1000	1.03	0
	100	1.08	0
	5	0.9	0
	1	1.03	0
∝-Methyl-D-glucoside	100	1.56	0
	5	1.43	0
Lactose	100	0.88	0
	5	0.87	0
Control	-	1.0	0

a Adherence ratio = $\frac{\%\ \text{adherence with carbohydrate}}{\%\ \text{adherence without carbohydrate}}$

TABLE XII

EFFECT OF HEPARIN, NANA[a], AND GANGLIOSIDE[b] ON ADHERENCE OF STRAIN 191 T1 *N. GONORRHOEAE* TO BUCCAL CELLS

Compound	Concentration $\mu g\ ml^{-1}$	Adherence[c] ratio	% inhibition
Sodium heparin	1000	0.99	0
	100	1.27	0
NANA	100	1.1	0
	5	1.05	0
Ganglioside	1000	0.2	80
	200	0.77	23
	5	1.05	0
Control	-	1.0	0

a N-acetylneuraminic acid.

b Bovine brain ganglioside type III.

c Adherence ratio = $\frac{\%\ \text{adherence with compound}}{\%\ \text{adherence without compound}}$

TABLE XIII

EFFECT OF LECTINS ON ADHERENCE OF STRAIN 191 T1 *N. GONORRHOEAE* TO BUCCAL CELLS

Lectin	Carbohydrate specificity	Concentration $\mu g\ ml^{-1}$	Adherence ratio[a]	% inhibition
Concanavalin A	D-mannose	100	0.95	0
		5	1.07	0
Peanut	D-galactose	100	1.48	0
		5	1.55	0
Soybean	N-acetyl-D-galactosamine	100	1.21	0
		5	1.17	0
Wheat germ	N-acetyl-D-glucosamine	100	0.81	0
		5	0.81	0
Control	-	-	1.0	0

a Adherence ratio = $\frac{\%\text{ adherence with lectin}}{\%\text{ adherence without lectin}}$

increased adherence by 50% and 20% respectively. Concanavalin A, specific for mannose, had no effect on adherence.

Effect of varying ganglioside concentration on adherence: Ganglioside inhibited adherence reproducibly at a concentration of 1000 μg ml^{-1}. Several other concentrations were tested and a graph of % inhibition vs. ganglioside concentration was constructed based in part on data obtained by Mr. Lian of our Laboratory (Figure 7). As the concentration of ganglioside increased the percent inhibition of adherence also increased up to a concentration of 1000 μg ml^{-1} above which no further inhibition was observed. Fifty percent inhibition was reached at a concentration between 200 and 300 μg ml^{-1}.

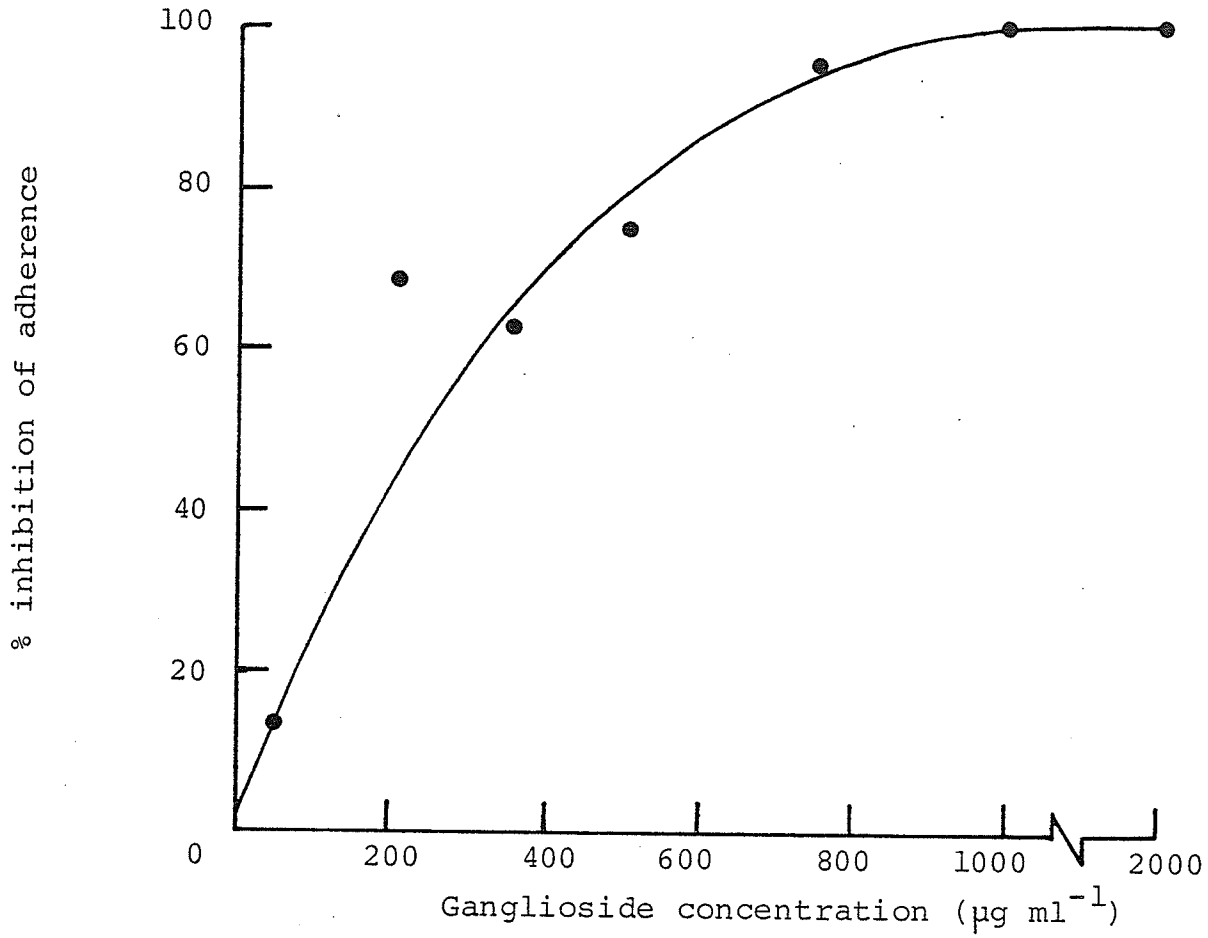

Figure 7. Relationship between ganglioside concentration and inhibition of adherence of strain 191 T1.

CHAPTER V

DISCUSSION

In the present study, growth of the gonococcus in ESP liquid medium was comparable to that found by Chan et al. (1975) and Allen (1978). The decrease in absorbance we observed in the stationary phase is in agreement with the findings of Lim et al. (1977), and supports findings of others regarding the instability and highly autolytic nature of the gonococcus under unfavorable conditions. Although others have found a decrease in the colony count during the lag phase, we observed no decrease in terms of absorbance. This agrees with the findings of LaScolea and Young (1974), Morse et al. (1974) and Lim et al. (1977).

Norrod and Williams (1979) found that PPS solution maintained the viability of gonococci for at least 60 minutes whereas simple solutions such as PBS, Gey's solution and saline would not. We also tested the viability of gonococci in PPS solution and MHBSS over 180 minutes and found no decrease for PPS solution but a two log decrease in viability for MHBSS. These results indicate the importance of the suspending solution in maintenance of the viability of the gonococcus.

Human buccal cells have been used as a model system in the study of adherence of various bacteria including Streptococcus species (Bartelt and Duncan, 1978; Alka et al., 1977; Gibbons and vanHoute, 1971), gram-negative bacilli (Johanson et al., 1979; Candy et al., 1978; Ofek et al., 1977; Ofek and Rottem, 1978), and N. gonorrhoeae (Punsalang and Sawyer, 1973; Swanson et al., 1974; Tramont and Wilson, 1977). We found that buccal cells could be

used as an alternative to tissue cultures of primary and continuous cell lines and would serve as a simple model in the study of gonococcal adherence. Buccal cells were used in this study because they are normal cells of the squamous type and easily obtained from the natural host of the gonococcus. Although cells from the buccal mucosa are physically distant from the typical site of gonococcal infection, the urogenital tract of humans, it is known that gonococci can colonize other mucosal sites of the human body as well. These include the conjunctiva, rectum, ear and pharynx. Continuous cell lines, which are abnormal, were avoided. Also, no completely suitable animal model is available for in vivo studies.

Gonococci have been labelled with ^{14}C by various authors for metabolic studies and also to investigate adherence (Swanson et al., 1974; Morse et al., 1974; Lim et al., 1977). Large quantities of ^{14}C-labelled compounds were used by Swanson et al. (1974), however they did not comment on the specific activity of the gonococci. Lim et al. (1977) found that the largest activity produced by ^{14}C-glucose occurred near the end of the exponential growth phase whereas ^{14}C-leucine produced the largest activity near the end of the lag phase. Morse et al. (1974) have shown that a relatively small percentage of the ^{14}C in ^{14}C-labelled glucose is actually incorporated into gonococci. In their study the largest percentage of ^{14}C was given off as respiratory CO_2 and was also released into the growth medium. Our data regarding the uptake of ^{14}C-glucose and ^{14}C-protein hydrolysate are in agreement with the findings of Lim et al. (1977). The specific activities we obtained for the gonococci were relatively low. This could be due to the low incorporation of ^{14}C as described

by Morse et al. (1974).

Swanson et al. (1975) have labelled gonococci with ^{125}I and found that relatively large activities could be obtained by this method. Their data show that the type 1 organisms labelled better than the type 4 and that specific activities varied on different labelling dates. The specific activities we reached by the direct labelling of gonococci with ^{125}I by the lactoperoxidase method are very close to those obtained by Swanson. We also found that the specific activities of gonococci varied and that type 1 labelled better than type 4. It is likely that the variations of surface protein available for iodination account for the fluctuating specific activities. Our observation that type 1 gonococci labelled better than type 4 is not unexpected since type 1 cells may have more available surface protein in the form of pili.

The findings of Parsons et al. (1979) show that there is a poor correlation between bacterial adherence assessed with radiolabelled bacteria and adherence assessed by visually counting the bacteria, in contrast with the findings of Sugarman and Donta (1979), who have shown that a good correlation exists between these two assay methods in their system. The reason for the discrepancy between the two groups is unclear. In our system type 4 cells showed adherence at nearly the same level as type 1 when radiolabelled gonococci were used. However, adherence of type 4, when assessed by both visual and viable counting methods, was negligible compared to type 1. Our findings agree with those of Parsons et al. (1979). We cannot give a clear explanation for the differences we found between these assay methods. The method of separating the unattached bacteria from the buccal cells may be

one factor involved in this difference. Schaeffer et al. (1979) found that separation on their membrane filters was ineffective because of entrapment of unattached bacteria. Similar filters were used in some of our experiments which could account for the differences observed.

Punslang and Sawyer (1973), Swanson et al. (1974, 1975), and Tramont and Wilson (1977) used human buccal cells and found that adherence of type 1 gonococci was much greater than type 4. Tramont and Wilson (1977) also found that adherence of type 3 and 4 cells of their strain was negligible (ie. 2% of buccal cells with 1 adhering gonococcus) in buccal cells from 10 donors. The results we obtained confirm these findings. None of our type 4 gonococci from seven strains adhered to the buccal cells. This could be due to a lack of corresponding adherence receptors on both buccal and type 4 gonococcal cells. Where conditions of adherence (ie. time and pH) were varied in our system, no adherence was observed for non-pilated cells. In contrast with the type 4 gonococci, type 1 cells from all six strains tested adhered to the buccal cells. There was also a variation in adherence from strain to strain which confirms the finding of Tramont and Wilson (1977). This suggests that adherence is a strain and type dependent phenomenon.

Mammalian cells and Gram-negative bacteria carry a net negative charge (Heckels et al., 1976; Jones, 1977). If surface charge plays a role in approach and adherence of the gonococcus, reduction in its negative charge as the pH approaches the pI could reduce the electrostatic barrier and enhance adherence. Mardh and Westrom (1976) have shown that a reduction in pH in their system enhanced adherence, with an optimum at 4.5. Johnson

and Osborn (1979) showed that adherence was greatest at a pH of 3.1 when compared to 6.8. Optimum pH values of 6.8 and 6.5 were determined by James et al. (1976) and Gubish et al. (1979) respectively. In the present study there was no increase in adherence over a pH range of 5.2-7.9 and no optimum was observed. That gonococci do adhere over a pH range suggests that something more than electrostatic force is involved in adherence.

Tramont and Wilson (1979) and Gubish et al. (1979) have shown that as the gonococci:eucaryotic cell ratio increased, adherence also increased to a point where saturation of the binding sites occurred. Saturation of binding sites on eucaryotic cells has been found in most bacteria as well (Gould et al., 1975; Bartelt and Duncan, 1978; Schaeffer et al., 1979: Pan et al., 1979). Buccal cells in our system also appear to have a limited capacity to adhere to gonococci, since an increment in the gonococci:buccal cell ratio increased adherence but led to no further change when saturation occurred. Our observations suggest that bacterial clumping was limited and may not play a major role in the observed increase. A continual increase in adherence would have been expected had this been the case.

James-Holmquest et al. (1974) used heat and formaldehyde to kill gonococci and found that there was no difference between the adherence of live or killed gonococci. Carruthers (1977) has found that viability is not required for the adherence of Vibrio parahaemolyticus to human fetal intestinal cells. She also showed that the method of killing the bacteria was important as it could alter the surface components responsible for adherence. This would make interpretation of the cause of inhibition difficult. Our observation that reduced viability

in the inoculum does not affect adherence agrees with the finding of James-Holmquest et al. (1974). Thus these observations suggest that adherence does not depend on active metabolism of the gonococcus.

Watt et al. (1978) used lotus, soybean, wheat germ, and castor bean agglutinins and found that these did not block the adherence of gonococci in their system. However they did find that castor bean agglutinin, which has an affinity for D-galactose, enhanced adherence. In our system Concanavalin A, wheat germ, soybean and peanut agglutinins also did not inhibit gonococcal adherence. However, like castor bean agglutinin in their (Watt et al., 1978) system, peanut agglutinin, which also has an affinity for D-galactose, and soybean agglutinin, which has an affinity for N-acetyl-D-galactosamine, enhanced adherence in our system. This increased adherence may be due either to the lectin bridging of carbohydrates on the buccal cell with those on the gonococcus and/or bridging of carbohydrates between gonococcal cells which could adhere in clumps to the buccal cells. This bridging is possible since lectins are known to have multiple binding sites (Sharon and Lis, 1972; Sharon, 1977). Blocking receptors with lectins in the gonococcus-eucaryotic cell system needs further investigation and could help to clarify the role of specific carbohydrates in adherence.

Ganglioside has been shown to bind toxins such as cholera, tetanus and botulinum (van Heyningen and King, 1976; Mellanby and Pope, 1976). Buchanan et al. (1978) found that ganglioside inhibited the adherence of purified gonococcal pili to human cells. The complete inhibition of gonococcal adherence by ganglioside (bovine type III) in the present study suggests

that only one primary adhesin facilitates this adherence. Ganglioside may function to block other adhesins as well. This suggests that an analogous structure on the surface of the buccal cell may act as the receptor. Since carbohydrate moieties of glycoproteins and glycolipids are exposed at the surface of eucaryotic cells (Marchesi et al., 1972; Costerton et al., 1977), it is likely that the carbohydrate portion of ganglioside or a similar glycolipid mediates their interaction with other molecules. In our system glucose, galactose, and N-acetylneuraminic acid, which are components of ganglioside, did not affect adherence when employed alone. This suggests that an oligosaccharide may be involved in adherence.

Buchanan et al. (1978) have suggested that ganglioside or a similar component on the human cell surface is likely the receptor for gonococcal pili. Although we found that ganglioside inhibited adherence of pilated gonococci to buccal cells we cannot determine from our data what role, if any, pili play in adherence.

CHAPTER VI

SUMMARY

1. Radiolabelling colonial types 1 and 4 of strain 191 N. gonorrhoeae with ^{14}C in ESP (enriched single phase) liquid medium resulted in very low specific activities even when competing substances in the medium were reduced.
2. ^{125}I labelling of strain 191 by the lactoperoxidase method gave relatively high specific activites compared to ^{14}C.
3. Proteose peptone saline solution, in contrast to modified Hank's balanced salt solution, maintained the viability of gonococci for at least 180 minutes at room temperature.
4. Assessment of adherence using radiolabelled gonococci showed that there was little difference in the adherence of non-pilated cells compared to pilated cells. However, assay of adherence by both visual and viable counting showed that adherence of non-pilated cells is negligible. Adherence of the pilated type, as assessed by visual counting, was lower than that obtained for the other two methods, which had similar values.
5. Increasing the gonococci:buccal cell ratio resulted in eventual saturation of the buccal cell receptor sites, beyond which no further adherence of gonococci occurred.
6. Detection of adherence after 15 minutes suggests that the adherence process is a relatively rapid phenomenon. It also appears to be time-dependent as an increase was observed when the time was extended to 60 minutes.
7. No significant difference in adherence was found when the pH of the suspending fluid was varied. This suggests that slight changes in surface charge which could occur as the pH varies have little effect on adherence.

8. The degree of viability of gonococci does not appear to affect their adherence to buccal cells.
9. Type 4 gonococci from seven strains showed no adherence when assessed by the viable count assay. However, type 1 gonococci from six of these strains were tested for adherence and all adhered to buccal cells but in varying degrees.
10. Concanavalin A, wheat germ, soybean, and peanut agglutinins did not inhibit adherence but soybean and peanut agglutinins enhanced adherence, possibly through bridging of gonococci and buccal cells.
11. The carbohydrates, D-glucose, D-mannose, D-galactose, alpha-methyl-D-galactoside, alpha-methyl-D-glucoside, lactose, heparin and N-acetylneuraminic acid failed to inhibit adherence of the 191 strain.
12. Ganglioside completely inhibited adherence at concentrations near 1000 $\mu g\ ml^{-1}$. This suggests that the ganglioside or a part of it is analogous to the receptor on the buccal cell or the adhesin on the gonococcus.
13. The viable count assay was shown to be the most suitable method for studying the adherence of N. gonorrhoeae to human buccal cells.

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APPENDIX A

GROWTH SUPPLEMENT

Formula per liter of distilled water:

Diphosphopyridine nucleotide, oxidized (Coenzyme 1)	0.25 g.
Vitamin B12	0.01
L-glutamine	10.0
Adenine HCl	1.3
Guanine HCl	0.03
p-Aminobenzoic acid	0.013
Cocarboxylase	0.1
Ferric nitrate	0.02
Thiamine HCl	0.003
L-Cysteine HCl	25.9
L-Cystine	1.1
Dextrose	100.0