The University of Manitoba

STUDIES WITH *NEISSERIA GONORRHOEAE*

by

Kwan-Leung Chan

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ABSTRACT

A new colony type of Neisseria gonorrhoeae, designated T1', has been isolated and characterized. It possesses pili and is virulent in chick embryos. A new liquid medium was devised and found to be suitable for the cultivation of type 1 (strain 3956) and types 1 and 2 (F62) cells incubated under 16% CO_2. Serological tests involving erythrocytes sensitized with gonococcal lipopolysaccharides demonstrated an extra antigenic determinant on type 1 lipopolysaccharide which is lacking on type 4 lipopolysaccharide. Hemagglutination of sensitized red cells in the presence of antiserum was inhibited by various sugars, especially lactose. Hence, galactosyl β1-4 glucose appears to be a part of type 1 and type 4 lipopolysaccharides.

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TABLE OF CONTENTS

PAGE

PAGE

LIST OF TABLES

LIST OF FIGURES

FIGURE PAGE

INTRODUCTION

In 1963, Kellogg and co-workers described four colony types of Neisseria gonorrhoeae based on the morphological characteristics of the organism grown on solid medium. After repeated subculture in vitro, types 1 and 2 retained virulence for male volunteers. Types 3 and 4, on the other hand, were avirulent.

Very little is known of the structural basis for this correlation between colonial appearance and virulence, and it is the purpose of this study to shed light on this relationship. Colonial morphology is a manifestation of surface structure of the individual organism, and since lipopolysaccharide is a surface component of N. gonorrhoeae, structural and immunological differences in the lipopolysaccharides isolated from the various colony types may exist and might serve to explain the relationship between virulence and colonial morphology.

LITERATURE REVIEW

Colonial Morphology

Since its first cultivation in 1882, *Neisseria gonorrhoeae* has been found to be very unstable on subculture, alterations of various properties such as nutritional requirements and antigenic structure, being observed. Atkin (1925) classified gonococci into two main types which he designated types 1 and 2. Type 1 was the smooth colonial variety whereas type 2 was rough. Morphologically, type 1 colonies on solid medium were large, irregular, flat, translucent and formed surface papillae. Type 2, on the other hand, formed small, round, raised colonies with uneven surfaces. The age of the culture used for identification varied from one week to one month. Kellogg *et al.* (1963,1968) showed that under specific conditions two forms could be differentiated according to colonial morphology. One formed small, convex and shiny colonies while the other formed large, flat and dull colonies. The small colonies were further differentiated into type 1 and type 2, the main difference being that type 2 colonies had slight internal granularity and a clear cut edge. Similarly, the large colonies were divided into types 3 and 4. Type 3 colonies were granular and of light brown coloration in contrast with type 4 colonies which were flat and colorless. Jephcott and Reyn (1971) observed an additional colony type (type 5). Type 5 formed shiny colonies which are very granular, opaque and with irregular edges. Kovalchik and

Kraus (1972) observed similar colony types in gonococci isolated from the rectum. In their study and that of James et al. (1973), considerable change in colonial morphology was induced by the presence of antimicrobial agents.

Properties of Colony Types

After the establishment of four colonial types by Kellogg's group, various properties were shown to be related to these types (Table I). One obvious conclusion that can be drawn from Table I is that the four types of gonococci fall into two groups. Types 1 and 2 form one group, while types 3 and 4 belong to another group. The distinction between the two groups is clear but the difference within the group is more subtle.

Other properties, such as fluorescent antibody fixation (Kellogg and Deacon, 1964), antibiotic susceptibility (Kellogg et al., 1963; Jephcott and Reyn, 1971), and nutrient requirements (Catlin, 1973) appear to be more related to strain than to colony type.

Relationship of Colony Types to Virulence

In his early work (1925), Atkin suggested that his type 1 was probably the most virulent form because this type was predominantly found in patients with acute urethritis, and type 2 was a less virulent form. However, direct correlation between virulence and morphological types was not established until the work of Kellogg's group. In human volunteers, type 1 and type 2 gonococci caused infection

TABLE I

PROPERTIES OF THE FOUR COLONY TYPES OF F62

Property	Colony Type 1	2	3	4	Reference
CO_2 requirement	grow better at higher CO_2 tension		-	-	Jephcott, 1972
Presence of pili	+	+	-	-	Swanson et al., 1971 Jephcott et al., 1971
Competence[a]	+	+	-	-	Sparling, 1966
Infectivity in man	+	+	-	-	Kellogg et al., 1963, 1968
Resistance to phagocytosis in vitro	+	+	-	-	Thomas et al., 1973 Thongthai and Sawyer, 1973
Auto-agglutinability in saline[b]	+	+	-	-	Kellogg et al., 1968 Swanson et al., 1971
Clumping[c]	+	++	+	-	Swanson et al., 1971
Host cell adhesion[d]	+	+	-	-	Swanson, 1973 Punsalang and Sawyer, 1973
Optimal disruption pressure (psi)	12,000	12,000	10,000	8,000	Martin et al., 1969
Direct Hemagglutination[e]	+	+	-	-	Punsalang and Sawyer, 1973
Presence in primary isolate	+	+	-	-	Kellogg et al., 1963, 1968 Sparling and Yobs, 1967 Jephcott et al., 1971

[a] Transformation to Streptomycin-resistance.
[b] Gonococci suspended in 1% formol-saline.
[c] A thin layer of fluid cultures of the organisms was examined by light microscopy. Clumping refers to cell aggregation observed under these conditions.
[d] Human cells, in vitro.
[e] Hemagglutination of rabbit erythrocytes by gonococci suspended in saline.

when inoculated into the male urethra; type 3 and type 4, on the other hand, were avirulent. They also observed that only types 1 and 2 were found in exudates of acute infection while types 3 and 4 appeared on subculture. The association of type 1 and type 2 with primary culture was later confirmed by Sparling and Yobs (1967), and Jephcott (1971).

However, enormous doses of type 1 and type 2 were required to cause infection whereas urethral pus from infected patients could readily infect volunteers. Watt et al. (1972) showed that subcultured type 1 and type 2 lacked a surface virulence factor which was present in gonococci growing in vivo, as subcultured types 1 and 2 were less resistant to bactericidal killing by normal human serum. They suggested that types 1 and 2 were avirulent phenotypically but still retained the genetic capability to become virulent under favourable conditions. Types 3 and 4, however, were genetically avirulent.

Early work with different animals failed to find a suitable species which could be used in the study of gonococcal infection (Hill, 1944). Miller et al. (1945) succeeded in producing a localized infection in the rabbit's eye by inoculating live gonococci into the anterior chamber. Lower primates are not susceptible to gonococcal infection, and immunosuppressed rats still retain resistance to experimental infection (Flynn, 1972). Arko (1972) successfully demonstrated in vivo cultivation of gonococci in rabbits by the use of surgically implanted chambers. Fresh isolates and type 1

organisms survived longer in these implanted chambers in the mouse (Flynn and Waitkins, 1973), but the difference in survival time is not large.

Tissue culture was also used in the study of the pathogenicity of gonococci. Generally, tissue cultures were destroyed by live gonococci, but no difference was detected between the virulent and avirulent types (Kenny and Avis, 1969; Ashton, unpublished data). Both type 1 and type 4 were ingested by the cells and protected from the lethal effect of bactericidal agents (Thayer et al., 1957; Kenny and Avis, 1969; Waitkin and Flynn, 1973).

Watt (1970) showed that phagocytosed gonococci were effectively killed in guinea pig and human polymorphonuclear leukocytes, but the organisms were more resistant to phagocytosis by human leukocytes. In 1973 Thongthai and Sawyer demonstrated that virulent types 1 and 2 have antiphagocytic cell surfaces. Therefore, they resisted phagocytosis while types 3 and 4 were phagocytosed readily. This finding was confirmed by Thomas et al. (1973). Swanson (1973) showed that type 2 organisms have a higher affinity for host cell surfaces and this attachment seems to be mediated by pili. The presence of pili on types 1 and 2 has been shown to impart antiphagocytic properties to the organisms, but antiphagocytic determinants other than the pili also seem to be present in these types (Punsalang and Sawyer, 1973).

The chick embryo has been extensively used in the study of gonococcal infection. The chorioallantoic membrane (CAM)

of 10-day embryos can be infected with N. gonorrhoeae (Bang, 1944). Serial allantoic passage in 8-day old embryos maintained and even reconstituted virulence of gonococci for volunteers (Walsh, 1963). Buchanan and Gotschlich (1973) reported that there was a significant difference in infectivity between the virulent types and the avirulent types when inoculated onto CAM of 10-day old chick embryos. This finding was not confirmed by Bumgarner and Finkelstein (1973). However, they found differences in the virulence of the Kellogg colony types of gonococci for chick embryos by means of intravenous inoculation of live gonococci. This difference in virulence was attributed to the relative clearance rate of the virulent and avirulent types in the blood of the chick embryo. This finding correlated with colonial virulence in volunteers.

Recently, experimental gonococcal infection was reproduced in chimpanzees (Lucas et al., 1971), and the infection appeared to be transmissible sexually (Brown et al., 1972).

Cultivation and Preservation of Gonococci

a) Solid medium: Neisseria gonorrhoeae is a nutritionally fastidious organism, requiring complex growth factors (Gould, 1944). Lankford (1950) described a chemically defined nutrient supplement for gonococcus culture media. It is made up of cocarboxylase, glutamine and dextrose. Kellogg et al. (1963) successfully identified and cultivated the four colony types of gonococci on solid medium made up of GC medium base

(Difco) with two % of Lankford's supplement. Recently the addition of one % isovitalex (BBL) in place of or in addition to Lankford's supplement has found wide use (Swanson, 1971; Punsalang and Sawyer, 1973; Thomas et al., 1973).

b) Liquid medium: Mueller and Hinton (1941) showed that culture media containing starch and casein hydrolysate or proteose-peptone were sufficient for growth of a number of freshly isolated gonococci. In the same study the growth of gonococci was shown to require CO_2. A chemically defined medium was devised by Welton et al. (1944) for the cultivation of the organism. Ten % CO_2 in air provided better growth than the atmosphere of the candle extinction jar, which contains about four % CO_2 but depends upon the numbers of plates contained in the jar. Not all strains grew equally well and 25% of the strains did not show any growth in this medium. Griffin and Racker (1956) demonstrated that the addition of small amounts of yeast extract abolished the CO_2 requirement of several strains of gonococci. The yeast extract itself was replaced by a mixture of hypoxanthine, uracil and oxaloacetate. Eighty % of the secondary cultures (not fresh isolates) grew in this medium in the absence of ambient CO_2, but with primary cultures only two out of 15 were able to grow in air. Tauber and Garson (1957) also found that yeast extract abolished the CO_2 requirement of gonococci, and they obtained good yield with a complex medium in the absence of CO_2. Use of an agar/liquid biphasic medium

permitted Gerhardt and Heden (1960) to obtain good growth of cells. Sparling (1966) claimed that stability of colony type was also achieved in the biphasic medium in addition to good growth. Brookes and Heden (1967) showed good growth of gonococci in a complex liquid medium. Type 4 colonies were used as the inoculum, and the colony type was stable in their medium. However, yeast extract was growth-inhibitory in the experiments. The CO_2 requirement was found to vary with different strains and increased aeration provided better growth.

Good growth and colony stability of type 1 were claimed to be achieved in cultivation of gonococci in a chemically defined protein-free liquid medium (Kenny et al., 1967). Hunter and McVeigh (1970) also devised a chemically defined medium. Stability of colony types in this medium was not alluded to. Moreover, not all strains grew in this medium. The presence of high CO_2 tensions provided better yields and the incorporation of a solid agar phase also enhanced growth.

Jephcott (1972) undertook to investigate three of the liquid media. He found that all three media permitted growth, but only the biphasic medium of Gerhardt and Heden (1960) was suitable in maintaining stability of type 1 and type 2 organisms. Furthermore, 10% CO_2 provided greater stability.

Catlin (1973) devised a chemically defined medium for gonococcal auxotyping. Good growth was reported but colony type stability in the medium was not investigated. Buchanan et al. (1973) used the meningococcal medium first devised by Frantz (1942), but again no detail on colony stability was

given.

c) Tissue and organ cultures: Gavrilescu et al. (1966) inoculated KB cell culture with gonococci, and observed abundant growth of the organism 24 hours after inoculation. Gonococci persisted for a long time (up to 88 days after inoculation). Killed KB cells did not support the growth of gonococci, nor did the tissue culture medium alone. Waitkins and Flynn (1973) demonstrated that type 1 gonococci were stable when inoculated onto mouse (3T3) and monkey (Vero and LLC-MK_2) cell cultures. Both types 1 and 4 showed intracellular multiplication. Carney and Taylor-Robinson (1973) demonstrated growth of type 4 gonococci on Fallopian tube organ cultures. Best growth was obtained when human tissue was used.

Both tissue and organ cultures seem to be suitable for the cultivation and maintenance of colony types of gonococci. In fact, they can be regarded as a form of biphasic media with cell monolayers or pieces of an organ in place of the agar as the solid phase. However, the complexity of these systems limits their wide use.

d) Preservation of gonococci: Type 1 and type 2 colonies are unstable, and on non-selective subculture type 3 and type 4 colonies appear gradually, becoming predominant (Kellogg et al., 1963,1968). Long-term preservation of gonococci is mainly achieved by freeze-drying and by storage in liquid nitrogen. Few studies have been done to determine the

survival rate and colony type stability after such treatments.

Brookes and Heden (1966) showed that the recovery rate of gonococci after freeze-drying was usually less than five % whereas storage of fresh isolates in liquid nitrogen after controlled rate freezing gave 20-30% survival. In 1971, Ward and Watt devised the "snap-freezing" method. Suspensions of gonococci in one % proteose peptone which contained eight % glycerol were frozen quickly and stored in liquid nitrogen. No detectable loss was observed on thawing and the colony types were stable.

Immunologic Aspects of the Gonococcus

a) Isolation of immunogenic fractions: Various antigenic preparations of gonococci have been introduced, largely aimed at providing a serological tool for detection of infection, but none of these has been entirely satisfactory. They range from antigenically multivalent whole cell preparations to the supposedly monospecific pili antigen (Table II).

In 1937, Casper divided gonococci into two serological types by comparative agglutination. He claimed that two type-specific polysaccharides were present. Stokinger et al. (1944) did not confirm Casper's finding, and found no evidence of distinct serological types.

In 1954, Chanarin used an alkali extract of gonococci to coat sheep erythrocytes in a study of the serological properties of many strains by means of hemagglutination, hemolysis and complement fixation techniques. The factor

TABLE II

ANTIGENIC PREPARATIONS FROM NEISSERIA GONORRHOEAE

Preparation	Composition and Characteristics	Reference
1. Whole cells		
i) live		Kellogg and Deacon, 1964
ii) heat-killed	heat-stable antigens	Deacon et al., 1959 Cohen, 1969
iii) formalinized	heat-labile antigen preserved	Deacon et al., 1959 Cohen, 1969
iv) alkali-treated		Chanarin, 1954
2. Cytoplasm		
i) whole		Reising and Kellogg, 1965
ii) antigen A	protein, M.W.≃ 200,000	Danielsson et al., 1969 Schmale et al., 1969
iii) antigen B	protein, very high M.W.	Lee and Schmale, 1970
iv) phenol-extracted antigen	protein (may be denatured)	Reising and Kellogg, 1965
v) Cheng's antigen	protein	Cheng, unpublished data
3. Endotoxin (LPS) extract		
i) phenol-water	mainly polysaccharide, some lipid and protein	Tauber and Garson, 1958 Maeland, 1968 Glynn and Ward, 1970
ii) aqueous ether	mainly protein, some lipid and polysaccharide	Maeland, 1969
iii) alkali	protein, lipid and polysaccharide; no endotoxic activity	Tauber and Garson, 1957 Maeland, 1966
iv) heat	protein, lipid and polysaccharide	Maeland, 1968
v) trichloroacetic acid	protein, lipid and polysaccharide	Boor and Miller, 1944 Maeland, 1968
vi) ethanol extract from allantoic fluid of infected chick embryo	degraded LPS	Chacko and Nair, 1966
4. Monospecific antigens		
i) β-antigen	glycoprotein	Apicella and Allen, 1973
ii) pili	protein	Buchanan et al., 1973 Punsalang and Sawyer, 1973

responsible for adsorption to erythrocytes was probably a polysaccharide. There were two distinct types of this antigen which were lost with the smooth-rough change. Maeland (1966) demonstrated the presence of reactive antibodies against erythrocytes sensitized with alkali extract in sera of infected and non-infected persons. In 1967, he further showed that antisera against whole cells gave four bands while antisera against erythrocytes coated with the alkali-extracted endotoxin showed only one band after immunodiffusion against live gonococci. In 1968, he showed the presence of two determinants ("a" and "b") in gonococcal endotoxin, and only determinant "a" was present in the phenol-water extract. Heat or alkali treatment was necessary for the sensitization of erythrocytes with an aqueous ether extract. Both determinants ("a" and "b") adsorbed onto the erythrocyte after these treatments. Antibodies against the endotoxin were mainly IgM and IgG. He also demonstrated that determinant "a" of the endotoxin contained six factors which determined antigenic specificity. These cross-reacted between strains, with meningococci and also with some other Gram-negative cocci. Determinant "b", on the other hand, seemed to be group specific to gonococci and meningococci, and showed no cross-reaction with other Gram-negative cocci. Galactose and lactose inhibited the reaction of antibodies with determinant "a". In 1971, he showed that a phenol-water extracted endotoxin of two of three strains studied also contained determinant "b" (Maeland and Kristoffersen, 1971).

In 1970, Hutchinson separated 181 strains of gonococci into five serological groups. Serological specificity, however, was lost after repeated subculture.

Glynn and Ward (1970) demonstrated that the main antigens concerned in bactericidal reactions are the lipopolysaccharide (LPS) and proposed that surface blocking antigens may restrict access to the LPS in the intact organisms. They also found a great degree of heterogeneity among the LPS (phenol extracted) from different strains. Sixty strains studied by them could be classified into four groups by their resistance to the bactericidal action of different antisera. Recently, Perry et al. (1973) provided evidence that LPS of type 4 organisms lacked an O-specific side chain whereas LPS of type 1 organisms possessed these side chains.

b) Host immune response to infection: Various investigators have observed the development of antibodies during gonococcal infection. Natural human antibodies, primarily IgG and IgM, have been shown to be reactive towards N. gonorrhoeae (Cohen, 1966,1967; Maeland, 1966). In infected patients, the level of the three major classes of immunoglobulins which are reactive with gonococci increased but only IgG present in the patient's serum combined with a heat-labile surface antigen (Cohen, 1969). Sera from patients with gonococcal infection as well as two-thirds of those from uninfected individuals had IgM antibodies against gonococcal surface determinants "a" and "b", while one-third of the patients' sera also

contained IgG antibodies reactive with the two determinants. Antibodies of this latter class were not found in sera from uninfected persons (Maeland, 1971). Cell-mediated immune responses to gonococcal infection were demonstrated by the lymphocyte transformation technique (Kraus et al., 1970; Grimble and McIllmurray, 1973). Kraus et al. also found that the cell-mediated response tended to increase in strength with two or more infections. Recently, Kearns et al. (1973) demonstrated that antibodies present in urethral exudates of male patients were largely secretory IgA. There seemed to be no difference in IgA titre with respect to the number of re-infections or the time of the onset of urethral discharge. In another paper, the same group of workers (Kearns et al., 1973) presented convincing data to suggest that men with repeated gonococcal infection typically have immune lymphocytes, serum antibodies and secretory IgA antibodies with antigonococcal activity. Patients with gonococcal infection also had elevated antibody levels against the purified pili antigen of gonococci (Buchanan et al., 1973). Kearns et al. (1973) have summed up the situation in the following words: "On the one hand, men with repeated episodes of gonorrhea, by definition lack resistance to disease. On the other hand, the same men have a variety of potential effectors that might eliminate the pathogenic organism but fail to do so."

Several suggestions have been put forward to explain this paradox, but experimental support for any of them is yet to come. One hypothesis is that the site of infection is

sequestered from the immune system. As a result, the invading gonococcus is not efficiently attacked by the defence mechanisms. Secondly, it may be a quantitative problem, i.e. the magnitude of the various immune responses is not great enough to eliminate the organism. Thirdly, the key virulence determinant may only be slightly immunogenic or not at all. Fourth, even if the 'protective antigen' is immunogenic, it may not be accessible to the immune mechanisms owing to the presence of blocking antigens on the bacterial surface. Finally, there could be a multitude of serological types of the organism such that immunity against one serotype does not necessarily protect against other serotypes.

Chemistry of the Surface Components of the Gonococcus

With electron microscopy, the surface architecture of the gonococcus was shown to be typical of Gram-negative bacteria (Swanson et al., 1971). It is made up of three layers; the plasma membrane, the intermediate layer and the outer membrane. While many meningococci (a closely related species) possess a polysaccharide capsule (Davis et al., 1973), so far capsules have not been convincingly demonstrated in gonococci. Recent evidence shows that types 1 and 2 have surface appendages which are made up of proteins. These surface appendages, or pili, are absent from the other colony types (Swanson, 1971; Jephcott et al., 1971).

a) Lipopolysaccharide (LPS): In 1944, Boor and Mennie (1950) extracted a glycolipid from gonococci by precipitation

of the cellular protein with trichloroacetic acid (TCA). Thomas and Mennie (1950) and Chanarin (1954) studied an alkali extract of gonococci, the nature of which was believed to be polysaccharide. In 1957, Tauber and Garson extracted endotoxin, which they felt to be protein, from gonococci by grinding and alkali treatment followed by precipitation with cold acetone. In another study, endotoxin was extracted by the hot phenol technique of Westphal et al. (1952). This endotoxin preparation was thought to be a lipopolysaccharide phosphoric acid ester (LPSP) on the basis of its chemical composition. They later modified the hot phenol extraction method and were able to show that a chemical relationship existed between endotoxins of gonococci and those of other gram-negative organisms. D-glucosamine, glucose and galactose were identified among the constituents of the LPSP preparation (Tauber and Russell, 1960) to which the lipid moiety was firmly bound (Tauber and Russell, 1961).

Maeland (1967) used an alkali extract of gonococcal endotoxin in his serological study. In a later study (1968), he prepared endotoxin by the following methods: a) heating in boiling water, b) alkali, c) TCA, d) aqueous ether, e) phenol-water. With the use of enzymes and periodate oxidation, he showed that the endotoxin was made up of two determinants, namely "a" and "b", as referred to earlier. Only determinant "a" was present in the phenol-water extract while the other extracts contained both determinants. In 1969, he went on to show that determinant "b" was a protein

and determinant "a" a polysaccharide. They probably formed a tightly bound complex on the bacterial surface. The chemical composition of the aqueous ether extract was mainly protein, carbohydrate, and lipid. The sugars identified were glucose, galactose, glucosamine and heptose. Sixteen amino acids were identified in the protein moiety, the main constituents being lysine, arginine, aspartic acid, glutamic acid, alanine, valine and leucine. In a subsequent study (1971), with phenol-water extract from the same strain and two other strains, he found the same sugars. In addition, 2-keto-3-deoxyoctulosonic acid (KDO) was also identified. The phenol extract contained about 11-14% of protein, while protein made up 83-88% of the aqueous ether extract. Maeland et al. (1973) found that a saline extract of gonococci contained the endotoxin complex (determinant "a" and determinant "b"). They suggested that two types of endotoxin may be present in gonococci, one being loosely bound and the other tightly bound.

From the above, it is clear that the word "endotoxin" refers to a substance released from the Gram-negative bacteria, in this case gonococci, by certain extraction procedures. It has been established that LPS is the moiety responsible for the biological properties manifested by endotoxin (Kadis et al., 1971), and as a result these two terms tend to be used interchangeably. However, it is also obvious that different extraction methods give products of different proportions of LPS and protein.

Repeated hot phenol extraction fails to rid the LPS preparation of protein. This is consistent with the proposal that LPS and protein form an outer membrane complex on the cell surface.

Relatively speaking, little is known with respect to the structural and serological properties of the LPS moiety isolated from different colony types of gonococci. Such studies are no doubt essential in understanding the pathobiology of this organism.

b) Appendages (pili): Gonococcal pili are filamentous protein structures which have been isolated and purified. They are immunogenic and by fluorescent antibody studies, are antigenically distinct from pili of meningococci, non-pathogenic Neisseria spp. and E. coli, while pili from types 1 and 2 gonococci are antigenically alike (Buchanan et al., 1972).

MATERIALS AND METHODS

A. Physicochemical Methods

Spectrophotometry: Ultraviolet absorption spectra of LPS preparations were obtained by the use of the Unicam SP 800B double beam recording spectrophotometer.

Infra red spectra of LPS preparations were obtained by using the Unicam SP 200 spectrophotometer. Freeze-dried LPS (1 mg) was pelletized with KCl (100 mg) for scanning. Alkali-treated LPS was neutralized with 0.2N HCl and dialyzed overnight against distilled water before freeze-drying.

Buffers: Phosphate buffer (0.01 M) pH 7.2, made 0.15 M with respect to NaCl, was used throughout this study.

Electron microscopy: The procedure of Swanson et al. (1971) was used in the examination of gonococci for pili. Gonococcal cells harvested from 20 hr. growth on GCBD plates were suspended in saline. A drop of this gonococcal suspension (10^{10} organisms per ml) was placed on a 200 mesh Formvar-coated copper grid. After 10 sec. the excess was extracted with filter paper. The grid was then washed for 10 min. with an 0.15 M sodium-potassium phosphate buffer (pH 7.0) by the method of Webb (1973). The grid was stained for 10 sec. with 2% uranyl acetate (pH 3.5). Observations were made with the Phillips EM 201 electron microscope.

KDO assay: 2-keto-2-deoxyoctulosonic acid (KDO) was assayed according to the method of Droge et al. (1970). Samples containing 0.01-0.05 µM KDO in 100 µl 0.25N sulphuric

acid were kept for 8 min. at 100°C. After cooling to room temperature, 50 µl 0.1N aqueous periodic acid was added, and the mixture kept at 55°C for 10 min. Then 200 µl 4% sodium arsenite in 0.5N hydrochloric acid was added, followed by 800 µl 0.6% aqueous thiobarbituric acid. After heating at 100°C for 10 min., the solution was shaken with 1 ml butanol containing 5% concentrated hydrochloric acid. The mixture was centrifuged at 1000 g for 1 min., and the absorbance of the butanol phase was measured at 548 nm. Since no standard initially was available, the estimate was based on that 1 µM KDO at 548 nm gave an absorbancy of 19.0 (Osborn, 1963).

Measurement of CO_2 tension: CO_2 concentration in growth studies was measured with the "Kwik-Chek" apparatus (Burrell Corp., Pittsburgh, Pa.).

Extraction of LPS: The gonococcal cells were grown in the ESP medium and treated with formalin (1% final concentration overnight at 4°C (Lindberg and Holme, 1972). LPS was prepared by the hot phenol method, described by Westphal and Jann (1965), from formalin-treated cells. Gonococcal cells (about 20 g. wet weight) were suspended in 200 ml of water at 68°C for 15 min., 200 ml of liquefied phenol (Fisher Scientific Company, laboratory grade) which was held at 68°C, were added. The suspension was stirred vigorously for 30 min. at 68°C. After cooling to 5°C in an ice bath, the suspension was centrifuged at 1500 g for 45 min. to facilitate the separation of the aqueous phase and the phenol phase. The clear aqueous phase was removed, while the phenol phase was added

to 200 ml of water at 68°C and a second extraction was performed. The aqueous phase was pooled, dialysed against running tap water for 48 hours, and then lyophilized. The solid recovered from lyophilization was dissolved in the least amount of distilled water which was just sufficient to dissolve all the crude material. Ultracentrifugation at 100,000 g for 4 hours was carried out three times to remove the nucleic acids and degraded products. The pellet was then redissolved in a small amount of distilled water and lyophilized.

Heat and alkali treatment of LPS: In the first, LPS was heated at 100°C for 1 hour. Alkali treatment involved the addition of an equal volume of 0.04 N NaOH to a solution of LPS (1 mg/ml). This mixture was neutralized by addition of 0.2 N HCl as described by Davies et al. (1958).

B. Bacteriological Methods

Maintenance of strains: Two strains of N. gonorrhoeae were used in this study. These were the F62 strain, obtained from the Centre for Disease Control, Atlanta, Georgia, and strain 3956, freshly isolated from a Winnipeg patient by the staff of the Manitoba Provincial Laboratory. The strains were grown on GCBD medium (Kellogg et al., 1963) which was made from Difco GC Medium Base with a 2% supplement of dextrose, L-glutamine and co-carboxylase. After growth on this medium for 20 hours at 37°C in a Hotpack CO_2 incubator under 5% CO_2 tension, the colonies were differentiated and selectively subcultured with the aid of the Bausch and Lomb Model 7

stereomicroscope. This microscope was equipped with a diffused, substage lighting source and top oblique illumination, as described by Kellogg et al. Colonial morphology described by Kellogg et al. and Jephcott and Reyn (1971) was followed in differentiating the colony types, as illustrated in Fig. 1 and Table III. Selective subcultures of colony types of the two strains were made daily.

Gonococcus strains were preserved in liquid nitrogen by the "snap-freezing" method of ward and Watt (1971). Strains were prepared for freezing by suspension in either 8% glycerol and 1% proteose peptone dissolved in water, or 10% dimethyl sulphoxide (DMSO) and 11.5% calf serum, dissolved in medium 199 (Grand Island Biological Co., Grand Island, N.Y.).

Viable counts: Viable counts of N. gonorrhoeae were obtained by the method of Miles and Misra (1938), using the GCBD medium (Kellogg et al., 1963) as the culture medium. Each of 12 drops per dilution containing 10-200 colonies were counted. Proportions of the colony types were also determined in each drop. However, the counts obtained were unreasonably high. In these experiments, a single pipette was used in preparing the various dilutions. A retrospective experiment showed that counts obtained by using a separate pipette for each dilution were much lower than those obtained by using a single pipette for all dilutions. The very high viable counts were therefore due to the use of a single pipette for all dilutions. This indicates that gonococcal

cells sticking onto the pipette could not be rinsed off readily. Despite this technical error, the findings from these experiments should still be valid since all the counts were performed in the same manner and the error would therefore be constant.

Growth of organisms in liquid medium: In this study, two liquid media were used; the first was biphasic medium (BP) of Gerhardt and Heden (1960) and the second, enriched single phase (ESP) medium. The biphasic medium is composed of 100 ml solid dextrose starch agar base (Difco) overlaid with 25 ml of dextrose starch peptone solution which contains Proteose Peptone No. 3 (15 g/l, Difco), dextrose (2 g/l), sodium chloride (5 g/l), and disodium phosphate (3 g/l). The ESP medium is composed of Difco GC medium base without agar and starch, Lankford supplement (5% v/v) and BBL Isovitalex (1% v/v).

Colonies of specific type (20 hr growth) were harvested and suspended in nutrient broth. One ml of this gonococcal suspension (10^8-10^9 CFU/ml) was used to inoculate flasks which contained the appropriate quantities of liquid media. The flasks were then placed in a New Brunswick Scientific Shaker-Incubator (Model R-25) and shaken at the rate of 160 rpm at 37°C for 30 hours. Compressed air and CO_2 were fed to the incubator at a controlled rate such that the final concentration was 16% CO_2 (see Results).

Clumping, hemadsorption and hemagglutination methods: Clumping tests were performed as described by Swanson et al.

(1971). Cells of specific colony type were grown in NCDM medium (Kenny et al., 1967) for 5 hours. A drop of the culture fluid was placed on a microscopic slide and covered with a cover slip. This was then examined by light microscopy for cell aggregation.

Hemadsorption and hemagglutination procedures were those described by Punsalang and Sawyer (1973). Hemadsorption was performed by flooding colonial growth of GCBD medium with a 3% rabbit erythrocyte suspension in saline. After 15 min. at 37°C, the surface of the medium was gently washed with saline and the colonies were examined under a light microscope for adherence of erythrocytes by reflected light. The hemagglutination technique was carried out by mixing 0.1 ml of a gonococcal suspension in saline (10^{10} cells/ml) with 0.1 ml of a 3% sheep or rabbit erythrocyte suspension in glass tubes (1 x 7 cm). A tube containing the erythrocytes alone was included as the control. The tubes were then sealed with paraffin and incubated at 37°C for 20 min. and then at 4°C for 16 hours. The settling pattern in each tube as compared with the control was examined for agglutination.

Chick embryo inoculation: Fertilized eggs were obtained from a single Leghorn flock reared in the Poultry Laboratory of this University. The chickens were fed on an antibiotic-free diet. The eggs were placed in a humid Single Stage egg incubator and incubated for 11 days. Intravenous inoculation of 0.1 ml volume of the gonococcal

suspension (1% proteose peptone saline was the diluent) was performed as described by Beveridge and Burnet (1946). For LD_{50} determinations, 11 eggs per dilution were inoculated, but 30 eggs per dilution were used in the time response-dose relation investigation. After inoculation, the embryos were examined daily by candling. Blood samples were withdrawn from chick embryos (dead or alive) according to Beveridge and Burnet, and tested for viable gonococci by plating onto GCBD agar. The inoculated plates were incubated for 20 hours at 37°C under 5% CO_2.

C. Immunological Methods

Production of antisera: Types 1 and 4 cells of strain 3956 grown in ESP medium as previously described were treated with formalin (final concentration 1% v/v) for 2 hours. These formalin-fixed cells were washed three times with sterile phosphate buffered saline and then suspended to a concentration of 10^{10} cells/ml in sterile saline. Two American Dutch female rabbits were inoculated intravenously with type 1 suspension, and one American Dutch female and one New Zealand white male rabbits with type 4 suspension, according to the immunization procedure given by Rudbach (1971). This method was, however, slightly modified to the extent that the inoculum size was increased ten-fold. Rabbits were inoculated intravenously with 10^8 organisms on days 0, 3 and 5. On days 7, 9 and 11, the inocula were increased to 10^9 organisms, and on days 14, 17 and 19 the

inocula were further increased to 10^{10} organisms. Two days after the administration of the last dose, the rabbits were bled from the heart. Their sera were separated from the red cells and stored at -20°C until required.

Glutaraldehyde fixation of red cells: Sheep red cells obtained from the National Biological Laboratory of Winnipeg were subjected to glutaraldehyde fixation according to the method of Bing et al. (1967). Packed sheep red cells which had been washed three times with phosphate buffered saline was chilled to 4°C in an ice bath. Glutaraldehyde solution which had been diluted to 1% (v/v) with sodium phosphate buffer and cooled to 4°C was added to dilute the red cells to 1-2% (v/v). The mixture was then incubated for 30 min. at 4°C with occasional mixing. The fixed red cells were collected by centrifugation at 1500 g for 20 min. They were then washed five times with saline and five times with distilled water. The fixed cells were finally suspended to a concentration of 30% in distilled water. Merthiolate was added to a final concentration of 1:10,000 (w/v) and the cells stored at 4°C.

Sensitization of red cells with LPS: Sheep erythrocytes, fixed or unfixed, were sensitized with LPS by the procedure described by Ciznar and Shand (1970). Sensitized red cells were kept as a 2% suspension (v/v) at 4°C. Merthiolate (1:10,000, w/v) was added only to the fixed cells as a preservative. The unfixed sensitized red cells could be kept for about 2 weeks without deterioration.

It was necessary to ensure that the sensitizing dose of LPS (1 mg/ml packed erythrocytes) was sufficient to achieve maximal sensitization. Thus, a second batch of erythrocytes was sensitized with the supernatant of the first batch of sensitized cells. Residual sensitizing activity was always demonstrated in the supernatant fluid, which indicated that the erythrocytes had been originally treated with an excess of LPS.

Tube agglutination: The preparation of antigen and direct tube agglutination titrations were performed as described by Hurvell et al. (1971) except that the time of autoclaving of the antigen was reduced to 90 minutes. Growth harvested from GCBD plates with saline was autoclaved at 120°C for 90 minutes. The cells were then washed three times with saline and diluted with saline to an optical density of 0.3 at 540 nm. Serial doubling dilutions of 0.2 ml of antiserum in phosphate buffered saline were made in tubes (1 x 7 cm), and 0.2 ml of the antigen was then added. The tubes were sealed with paraffin and incubated for 14 hours in a water bath held at 52°C. Agglutination was read by the resuspension technique with the aid of a magnifying lens. The end point was the highest dilution of antiserum which gave definite agglutination under these conditions.

Complement fixation: Serial doubling dilutions of 25 µl of heat inactivated serum (56°C for 45 minutes) were made in veronal buffered saline in "Microtiter" U-bottom

plates (Mitrobiological Associates). Final dilutions ranged from 1/8 to 1/1028. Equal volumes of antigen ($5x10^8$ cells/ml), prepared by Hurvell's method, were added, followed by 25 µl of complement adjusted to contain 2.5 hemolytic units. After incubation at 37°C for 1 hour, 25 µl of 5% sensitized red cells were added and the plates shaken and left at 37°C until lysis was complete in the control. The plates were then read after centrifugation at 600 g for 5 minutes. The end point was taken as the highest dilution of antiserum which caused 50% hemolysis.

Absorption of antisera: (a) With whole cells killed by heat, 0.4 ml antiserum was mixed with sediment from 40 ml of Hurvell's tube agglutination antigen (OD_{540}=0.3). The mixture was incubated at 37°C for 2 hours before centrifugation.

(b) With formalin-treated whole cells, 0.15 ml of antiserum was absorbed with 0.2 ml packed cells and incubated for 2 hours at 37°C followed by further incubation at 4°C for 30 hours.

(c) Antiserum (0.8 ml) diluted two-fold was also absorbed with unsensitized sheep red cells (0.2 ml packed cells) and incubated at room temperature, about 20°C, for 20 minutes.

(d) With sensitized sheep red cells, 0.15 ml antiserum was mixed with sediment from 8 ml of a 2% suspension of the red cells, and incubated at 37°C for 2 hours.

In any of these procedures, absorption was repeated if there was evidence of incomplete absorption.

Passive (indirect) hemagglutination: Microtiter techniques with U-bottom plates were more satisfactory than those with tubes (1 x 7 cm) or V-bottom plates. The optimal sensitized erythrocyte concentration for visualization of the reaction was 0.5%. The procedures described by Tomiyama et al. (1973) were followed, except that the plates were read after overnight incubation at 4°C. The end point was taken as the highest dilution of the serum which gave distinct agglutination of the red cells, expressed as hemagglutination (HA) unit per 25 µl. Phosphate buffer saline was used as the diluent.

Passive (immune) hemolysis: This was performed in a manner similar to the hemagglutination test. After addition of sensitized cells to serial dilutions of the antiserum in phosphate buffer saline, the plates were incubated at 37°C for 30 minutes, after which 25 µl of complement containing at least 8 hemolytic units ($C'H_{50}$) was added. After further incubation at 37°C for 30 minutes, the plates were centrifuged at 600 g for 5 minutes. The titre was the reciprocal of the highest serum dilution which showed 100% hemolysis. The following controls were always included:

(i) unsensitized red cells, antiserum (1/8 dilution) and complement,

(ii) sensitized red cells and complement, and

(iii) sensitized red cells and antiserum (1/8 dilution).

Hemagglutination inhibition by LPS: Serial dilutions of LPS were prepared ranging from 0.195 to 6.250 µg in 25 µl. Three HA units in a 25 µl volume of antiserum were used. The antiserum had been heat inactivated and preabsorbed with unsensitized erythrocytes. After incubation of the plates at 37°C for 1 hour, 25 µl of sensitized erythrocytes were added. The plates were read after further incubation for 1 hour at 37°C and then for 2 hours at room temperature. The lowest LPS concentration which gave total inhibition of hemagglutination was determined.

Hemagglutination inhibition by sugars: The sugar (25 µl of 0.28 M solution in distilled water) was added to two-fold dilutions (25 µl) of inactivated antiserum which was initially absorbed with unsensitized erythrocytes (Maeland, 1969b). After incubation at 37°C for 1 hour, 25 µl of sensitized erythrocytes (0.5%) were added and the suspension shaken. The titres were determined after incubation at 37°C for 1 hour followed by a period of holding at room temperature for 2 hours, in a manner similar to the passive hemagglutination test.

Immunodiffusion: The procedures of Hurvell (1972) were followed. However, in this study the wells were 3 mm in diameter, 6 mm apart (centre to centre), and the diffusion was performed at room temperature.

All immunological tests were performed in duplicate.

D. Statistical Methods

Mean lethal dose (LD_{50}) determination: Probit analysis of the data was performed on the computer according to the Teletype programs set up by the Health Sciences Computer Department of this University. The LD_{50} values derived from such analyses were also determined by the computer.

Response-dose relationship: In this case, time of death was used as a response. The analytical procedures have been described by Eckert et al. (1954) and Bryan (1956). Several transformations were tried, and the logarithm of time was the most suitable time metameter. The mean time of death in this analysis was termed 'median latent period' which is the time required for 50% of the chick embryos to show the desired response; in this case, death.

RESULTS

Description of Colony Types

In classifying the colony types, we encountered difficulties in the identification of T1 and T2 because of the presence of an extra distinct small and shiny colony. On careful and repeated examination, this type appeared to be too granular and possessed an edge which was too irregular to fit the description by Kellogg *et al.* (1963,1968) of T1 and T2. This new type is not an intermediate form, as it is stable and has been carried in our laboratory for six months. This observation therefore led us to define a new colony type which we have termed T1'. The T1' colony is darker and much more granular than the other virulent types 1 and 2, and has an irregular edge, although it is of the same size.

Six different colony types were observed for each of the two strains 3956 and F62. The colonial morphology of the six types is essentially the same for the two strains. Strain 3956, however, tends to form slightly larger colonies than F62.

The six colony types are T1, T1', T2, T3, T4 and T5. The morphological characteristics of T1, T2, T3, T4 and T5 are consistent with the observations of Kellogg *et al.* (1963,1968) and Jephcott and Reyn (1971), but T1' has not been described in the literature. The six colony types

of 3956 are illustrated in Fig. 1 and their main morphological features are summarized in Table III.

Some physical properties of the colony types were investigated and are shown in Table IV. Electron microscopy demonstrated that pili are present on T1, T2 and T1' (Fig. 2). The morphology of the pili on the three piliated colony types is identical, and very often the pili were in bundles as previously described by Jephcott et al. (1971).

Conversions from one colony type to another were observed at initial selective subcultures, and the following were most commonly noted:

T1 → T4,
T2 → T3,
T1' → T5, and
T3 → T4.

However, after about 60 selective subcultures, the strains were well adapted and gave 99% original colony type upon further transfers. Subsequent studies were carried out on these stabilized types.

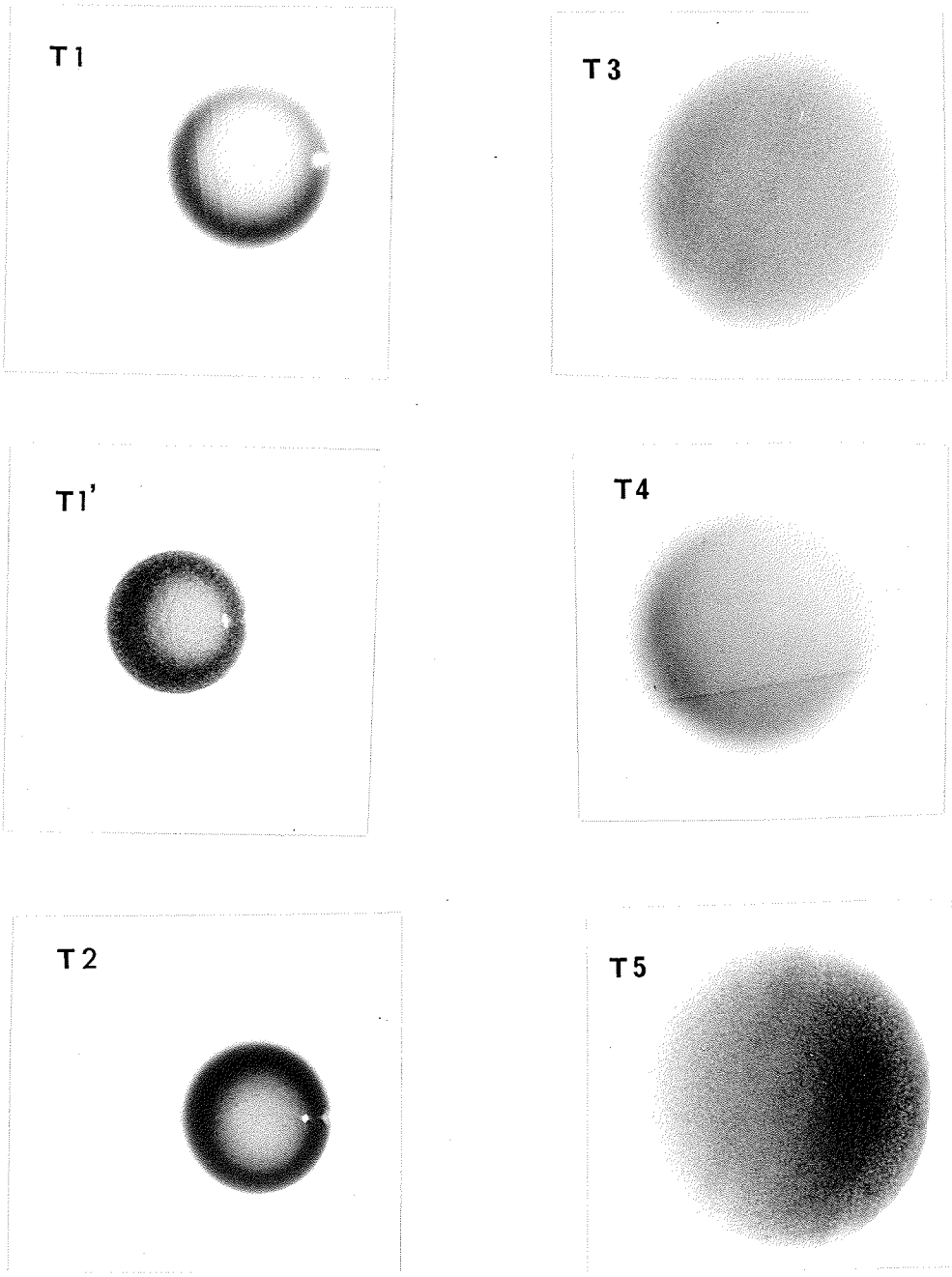


Figure 1. Pictures to illustrate the colonial morphology of colony types of strain 3956. (x 30)

TABLE III

COLONIAL MORPHOLOGY OF STRAIN 3956*

Characteristic	Colony Type					
	T1	T2	T1'	T3	T4	T5
Diameter (mm)	0.7	0.7	0.7	1.4	1.4	1.4
Coloration	grey gold	dark gold	very dark gold	pale brown	colorless	brown
Elevation (convexity)	++	++	++	+	+	+
Surface	smooth	smooth	uneven	smooth	smooth	uneven
Reflection of light	+	++	++	-	-	+
Granularity	-	+	++	+	-	++
Edge	smooth	smooth and well-defined	irregular	smooth	smooth	irregular
Opacity	translucent	translucent	opaque	translucent	translucent	opaque

*Observed under oblique transmitted light. Grown on GCBD medium for 20 hr.
- to ++ = Various degrees of expression of the character.

TABLE IV

PHYSICAL PROPERTIES OF COLONY TYPES OF STRAINS 3956 AND F62*

Property	Colony Type T1	T2	T1'	T3	T4	T5
Saline Auto-agglutinability	+	+	++	-	-	++
Viscidity	++	+	-	++	++	-
Clumping	+	+++	ND**	+	-	ND
Hemadsorption	+	+	+	-	-	-
Hemagglutination:						
3956 + Rabbit erythrocytes	±	+	±	-	-	+
3956 + Sheep erythrocytes	++	++	+	-	-	+
F62 + Sheep erythrocytes	±	+	++	-	-	-
Pili 3956	+	+	+	-	-	-
F62	+	+	+	-	-	-

*- to +++ = Various degrees of expression of the character.
**ND = Not done.

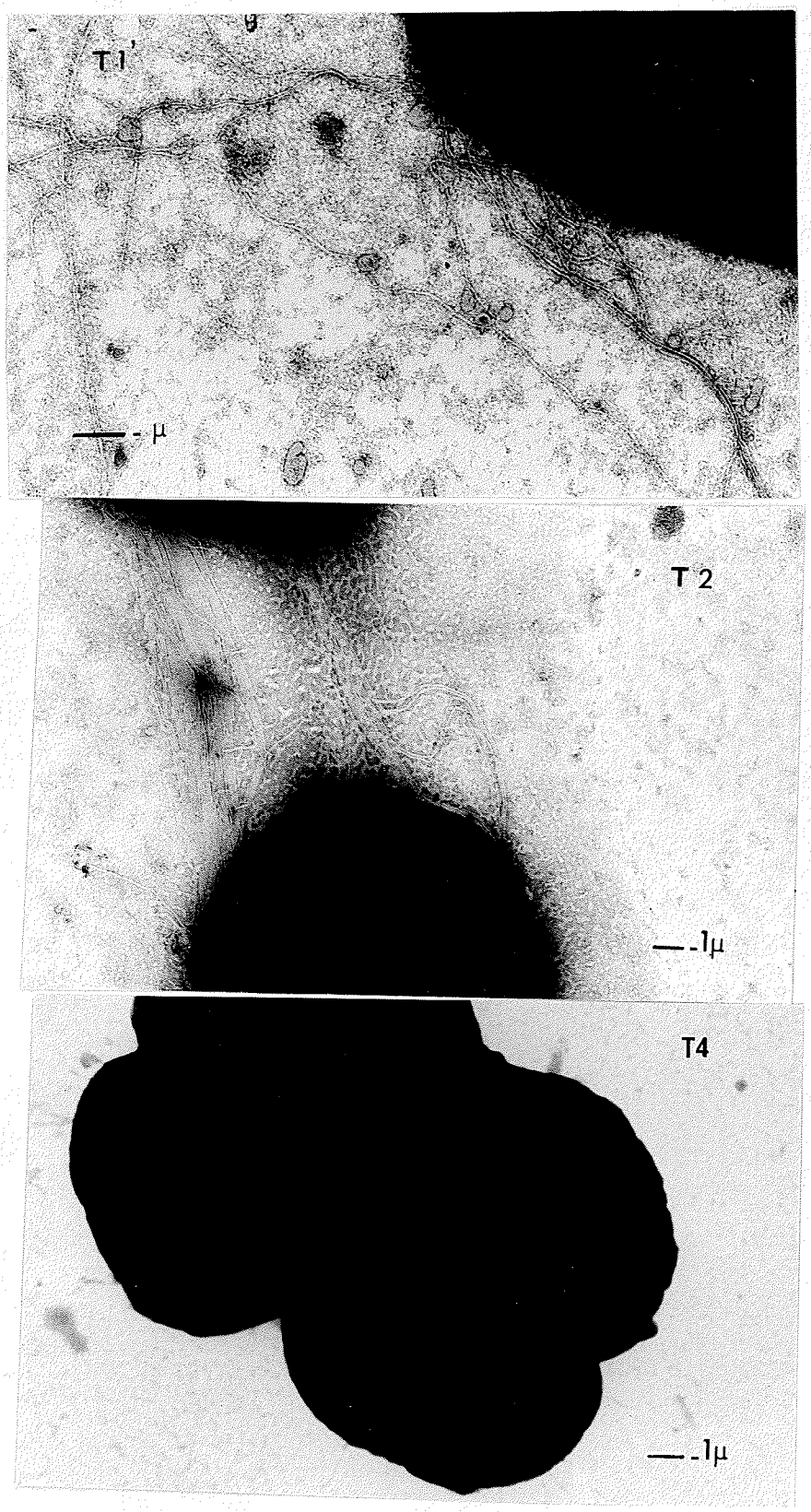


Figure 2. Electron micrographs to demonstrate pili in colony types of strain 3956.

Preservation of Gonococci

There is no large difference in viable counts on the two media tested (Table V). It appears that both glycerol and DMSO serve very well as a protective agent during the freezing process. On the whole, the viable counts of T1 and T4 in both media actually increased after thawing. The same phenomenon was also observed by Ward and Watt (1971). Moreover, greater than 95% colony type stability was made possible by this 'snap-freezing' method in both media. This means that counts of colony types recovered after thawing were greater than 95% of the original type.

Cultivation of Gonococci in Liquid Medium

For the comparison of the two media, 250 ml flasks containing 25 ml of the ESP medium or biphasic medium were inoculated with gonococcal suspensions (0.5 ml) prepared from specific colony types. Before inoculation, the flasks had been incubated overnight at the same CO_2 tension which was to be used in the experiment. The inoculated flasks were then incubated at 37°C and constantly shaken at a rate of 160 rpm. The CO_2 and air were premixed before feeding into the shaker-incubator and the CO_2 tension was adjusted by the regulator fitted onto the CO_2 cylinder. Viable counts were performed at fixed intervals.

The effect of CO_2 on the growth and type stability of T1 (3956) in BP and ESP media is shown in Figs. 3, 4, 5 and 6. These figures were constructed from the data derived from

TABLE V

PRESERVATION OF STRAIN 3956 (TYPES 1 AND 4) BY THE SNAP-FREEZING METHOD

Time of cell count	Viable cell count (CFU/ml)*			
	T1		T4	
	8% glycerol	10% DMSO**	8% glycerol	10% DMSO
Before freezing	1.7×10^6	2.5×10^7	2.8×10^6	2.0×10^6
1 day after freezing	4.3×10^6	1.6×10^6	3.6×10^7	5.1×10^6
1 month after freezing	3.6×10^6	4.6×10^6	3.0×10^7	7.2×10^5
3 months after freezing	2.6×10^7	6.1×10^7	1.4×10^8	2.2×10^7

*Both colony types showed >95% stability after freezing and thawing. CFU = Colony forming unit.
**Dimethylsulphoxide.

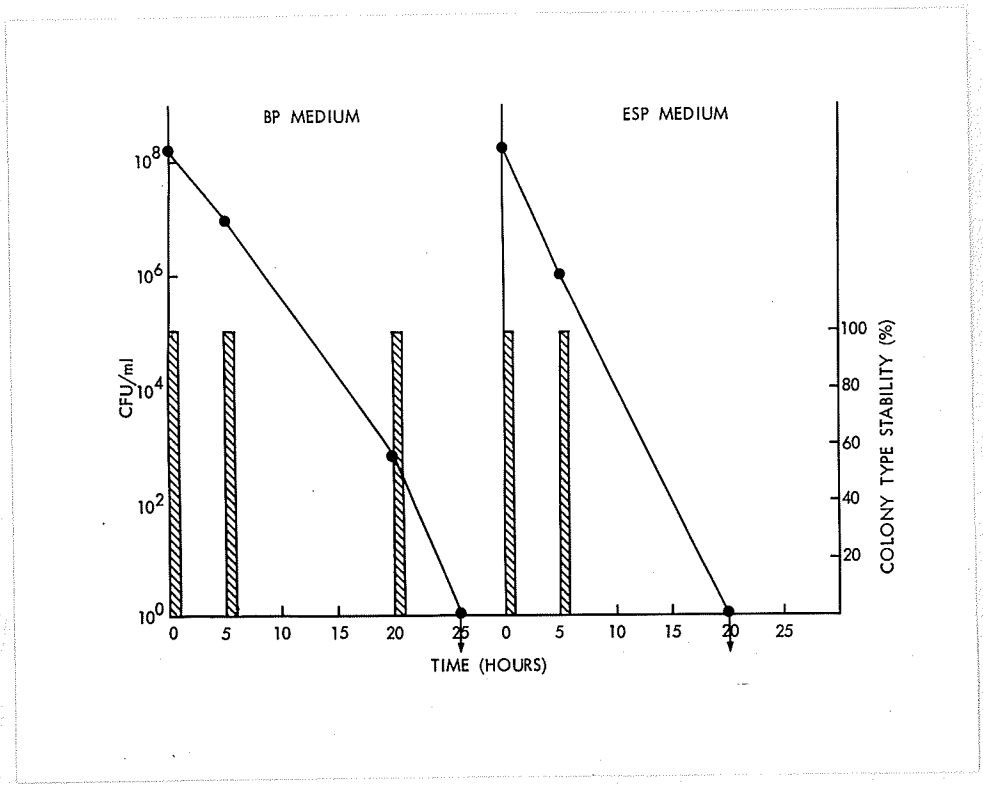


Figure 3. Comparison of growth and colony type stability of cells from type 1 (strain 3956) colonies in BP and ESP media in air (0.03% CO_2).

●—● = viable cell count. The bar graph shows the colony type stability. ▨ = original colony type. □ = altered colony type.

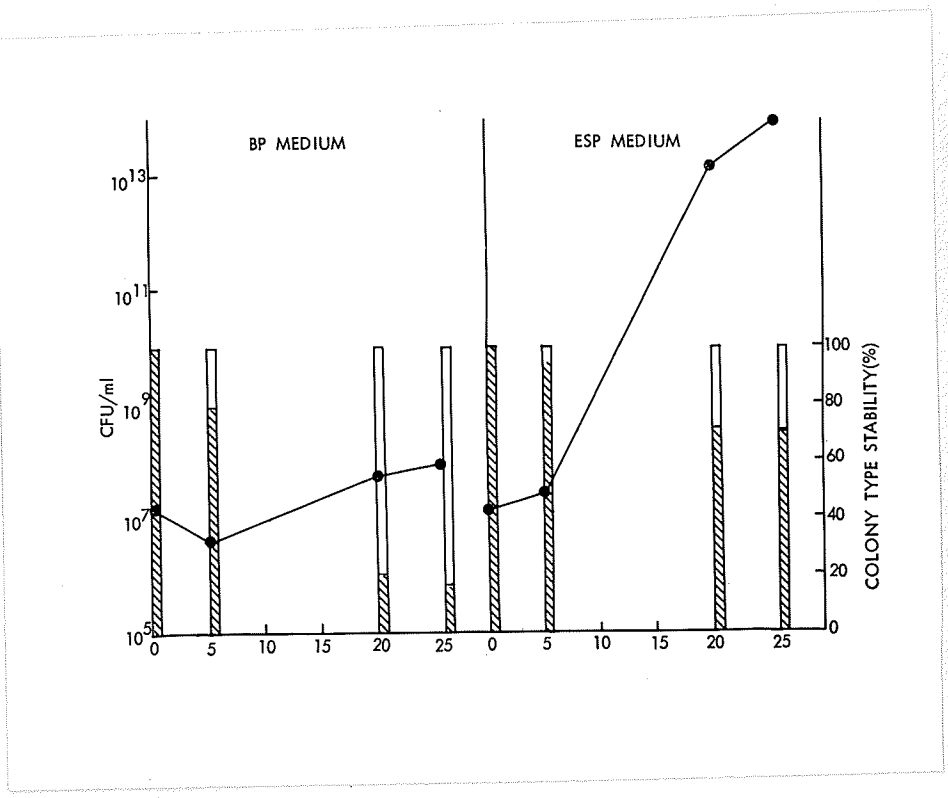


Figure 4. Comparison of growth and colony type stability of cells from type 1 (3956) colonies in BP and ESP media at 6% CO_2.

⊙—⊙ = viable cell count. The bar graph shows the colony type stability. ▨ = original colony type. □ = altered colony type.

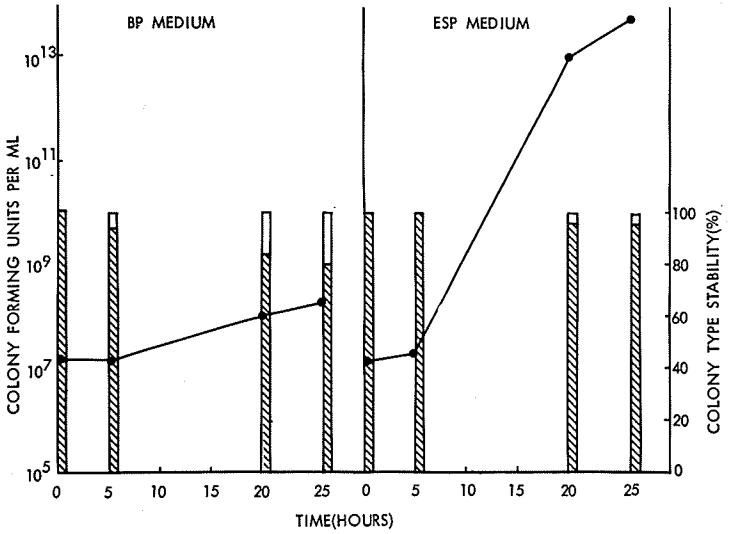


Figure 5. Comparison of growth and colony type stability of cells from type 1 (3956) colonies in BP and ESP media at 12% CO_2.

⊙—⊙ = viable cell count. The bar graph shows the colony type stability. ▨ = original colony type. □ = altered colony type.

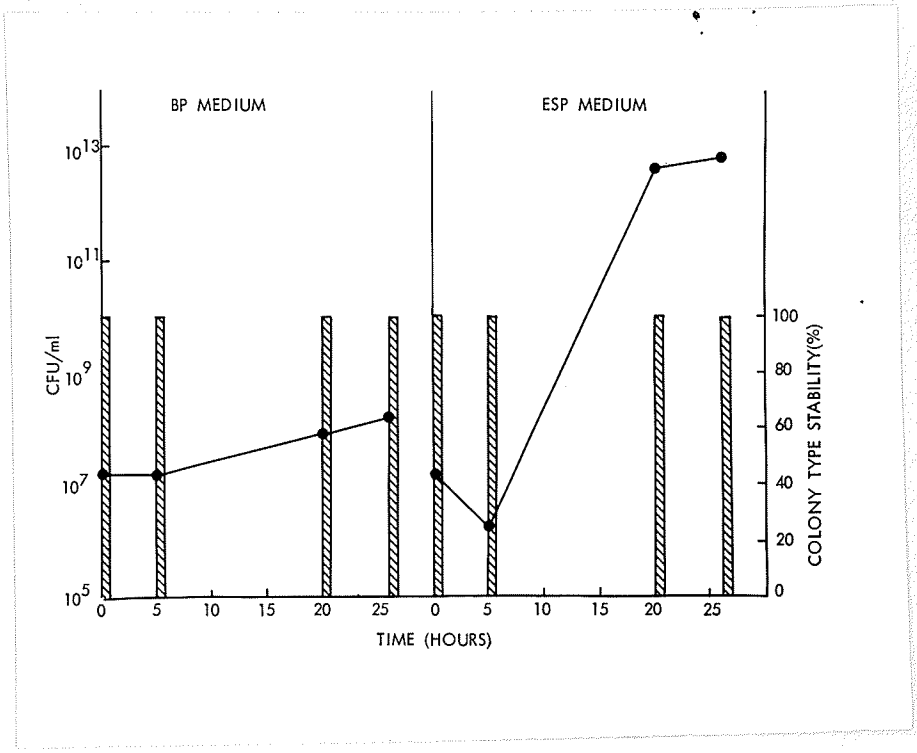


Figure 6. Comparison of growth and colony type stability of cells from type 1 (3956) colonies in BP and ESP media at 16% CO_2.

●—● = viable cell count. The bar graph shows the colony type stability. ▨ = original colony type. ▭ = altered colony type.

single experiments. At 0% CO_2, there was no growth in either medium. The death rate, however, was slower in the BP medium since viable cells could still be recovered 20 hours after inoculation. This suggests that the BP medium may be useful for short term preservation of the organism. Moreover, the viable count also showed that the colony types do not change when no multiplication occurs. Growth was obtained in both media at 6%, 12% and 16% CO_2 tensions. In all cases, the ESP medium gave a much better yield than the BP medium. The colony type stability was dependent on CO_2 tension, as it increased with the increase of CO_2 tension from 6% to 16%. At 16% CO_2, 100% stability was obtained in both media. At 6% and 12% CO_2 tensions, the ESP medium provided a higher degree of type stability than found in BP medium.

Cultivation of T1 (F62) in the ESP medium at 12% CO_2 gave good yields and 100% type stability. Under the same conditions, T2 (F62) also grew well and was stable (Fig. 7).

On the other hand, for T2 (3956), CO_2 tensions as high as 16% failed to provide a significant degree of colony type stability in the ESP medium (Fig. 8). This strain difference in terms of nutrient and CO_2 requirements is consistent with the observation of Catlin (1973).

At 0% CO_2, T4 (3956) grew in both media, but the yield was improved at 12% CO_2 (Fig. 9). Under 0% and 12% CO_2, colony type stability was obtained in both media. This finding is compatible with the observation that T4 is the most stable type.

A summary of yield and type stability in both media

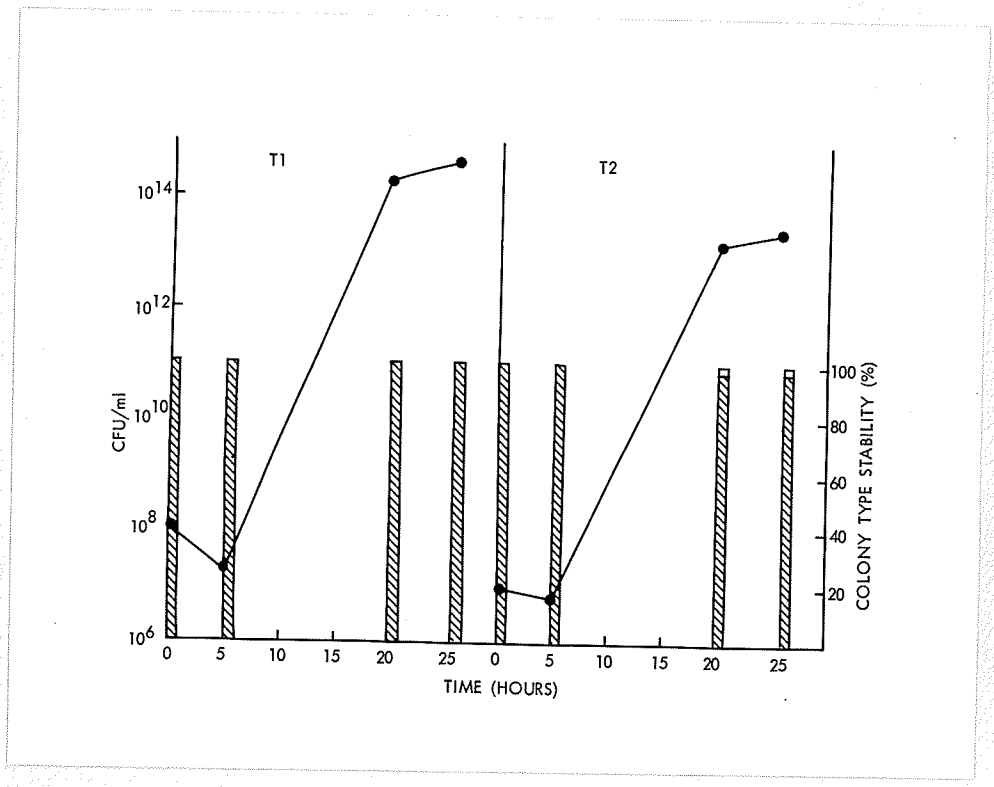


Figure 7. Growth and colony type stability of types 1 and 2 (F62) cells in ESP medium at 12% CO_2.

●—● = viable cell count. The bar graph shows the colony type stability. ▨ = original colony type. □ = altered colony type.

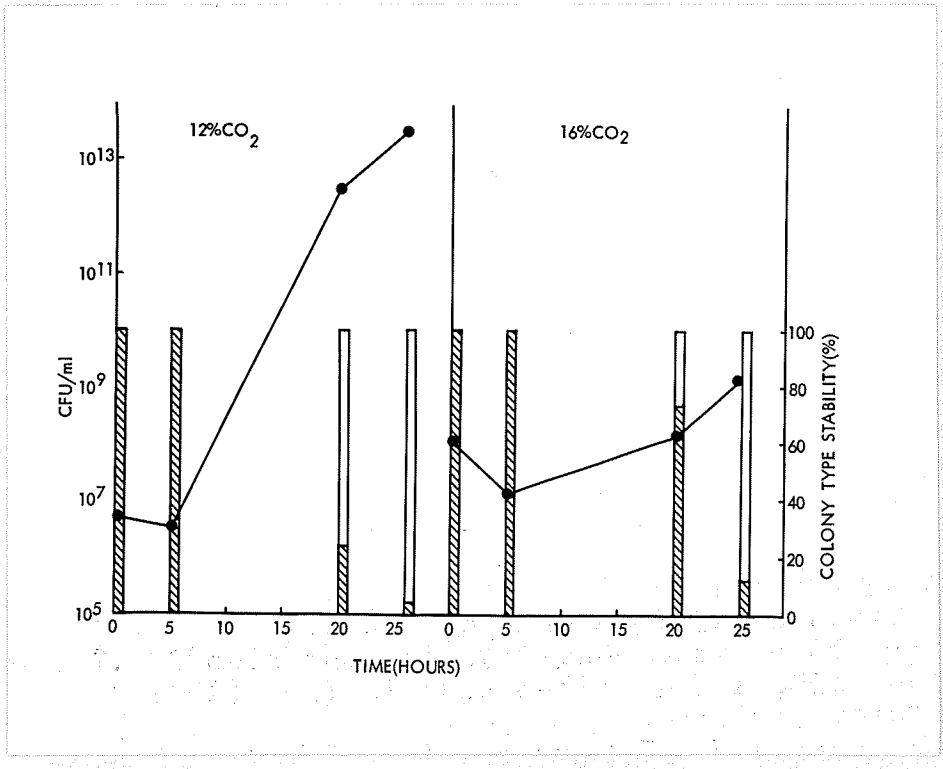


Figure 8. Growth and colony type stability of cells from type 2 (3956) colonies in ESP medium at 12% and 16% CO_2 tensions.

●—● = viable cell count. The bar graph shows the colony type stability. ▨ = original colony type. □ = altered colony type.

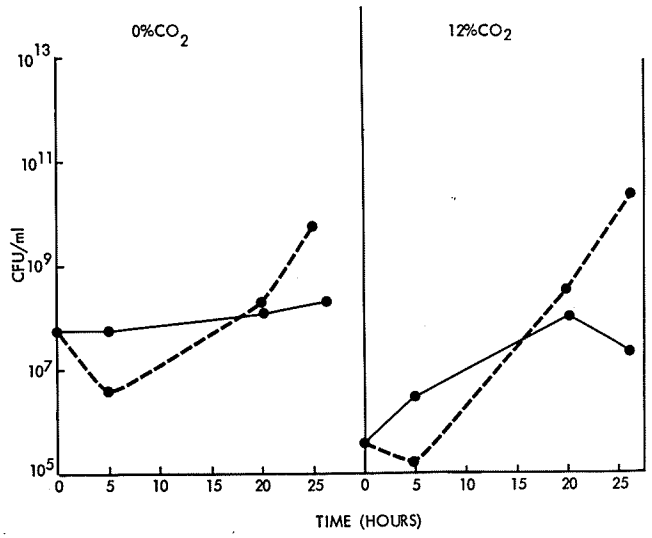


Figure 9. Comparison of growth of cells from type 4 (3956) colonies in BP and ESP media in air and at 12% CO_2 tension.

●---● = viable cell count of the ESP medium. ●—● = viable cell count of the BP medium. In both media at 0% and 12% CO_2, 100% colony type stability of type 4 (3956) was observed.

after 27 hr. incubation is given in Table VI.

Conversions of T1 to T4 and T2 to T3 were also observed in liquid medium.

Relative Virulence of Colony Types

Bumgarner and Finkelstein (1973) have shown that death of 11-day chick embryos inoculated intravenously with live gonococci parallels virulence studies with human volunteers performed by Kellogg et al. (1963,1968). Types 1 and 2 were more virulent (lower LD_{50}) than types 3 and 4.

Mortality incidence responses for all dose groups of the various colony types were determined (see Appendix). These data were subjected to probit analysis. The probit-log dose regression lines for the various colony types (except T1' because even the highest dilution used killed almost all of the chick embryos inoculated, so that a probit analysis could not be carried out) from two sets of experiments are shown in Fig. 10. Live T1 and T2 cells are significantly more virulent than T3, T4, T5 and heat killed T1 and T4, the probit lines of which are close to each other.

The LD_{50} values were determined by taking log dose values which correspond to a probit value of 5, and the calculations for the various types are shown in Table VII. Types 1, 2 and 1' have much lower LD_{50} doses than the other types, and heat killed T1 cells had a much lower LD_{50} value than live T1 cells.

Variation in the LD_{50} doses determined in separate

TABLE VI

SUMMARY OF EFFECT OF CO_2 TENSION ON THE GROWTH AND STABILITY OF GONOCOCCUS TYPES IN TWO LIQUID MEDIA

Colony Type and Strain	% CO_2	Inoculum size (CFU)	Viable count after 27 hr. incubation in Biphasic medium CFU/ml	Biphasic medium % type stability	Enriched single phase medium CFU/ml	Enriched single phase medium % type stability
T1 (3956)	0	$2x10^8$	0	-	0	-
	6	$1x10^7$	$1x10^8$	16	$1x10^{14}$	70
	12	$2x10^7$	$3x10^8$	80	$7x10^{13}$	96
	16	$2x10^7$	$2x10^8$	100	$8x10^{12}$	100
T1 (F62)	12	$1x10^8$			$6x10^{14}$	100
T2 (3956)	12	$7x10^6$			$5x10^{13}$	4
	16	$1x10^8$			$2x10^9$	12
T2 (F62)	12	$1x10^7$			$4x10^{13}$	98
T4 (3956)	0	$8x10^7$	$3x10^8$	100	$8x10^9$	100
	12	$7x10^5$	$4x10^7$	100	$4x10^{10}$	100

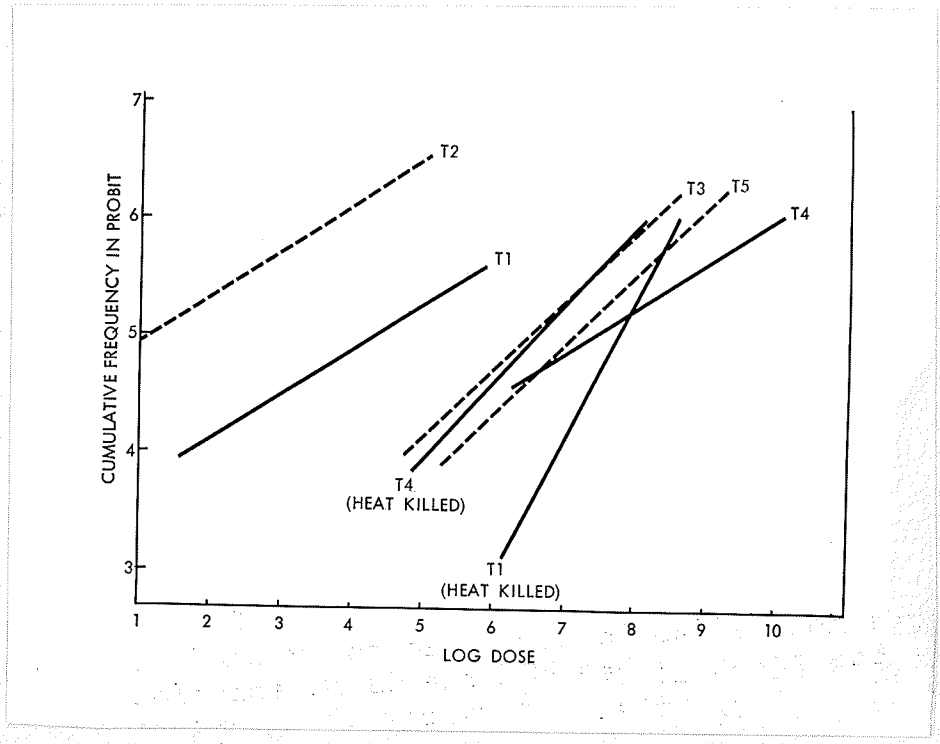


Figure 10. Mortality incidence of 11-day old chick embryos three days after intravenous inoculation with gonococci (strain 3956).

(——) represents experiments performed at the same time. (---) represents another set of experiments performed at a different time.

TABLE VII

VIRULENCE OF COLONY TYPES OF STRAIN 3956 FOR 11-DAY CHICK EMBRYOS INOCULATED INTRAVENOUSLY

Colony Type	LD_{50} (CFU) at 72 hr.*
T1	1.82×10^{4a}, 6.10×10^{3c}
T1 (heat killed)	5.19×10^{7a},
T4	1.82×10^{7a}, 4.44×10^{6c}
T4 (heat killed)	4.13×10^{6a}
T2	1.22×10^{b}
T1'	$< 8.5 \times 10^{2b}$**
T3	2.99×10^{6b}
T5	1.16×10^{7b}

*Determined by probit analysis.
a,b,c, denote experiments performed at different times.
**A precise value could not be obtained, because even the smallest dose used in the experiment killed almost all the chick embryos.

experiments was observed. Probit analysis of relative lethality in two separate experiments (Fig. 11) showed that the difference between T1 and T4 remains essentially unchanged despite the variation in the LD_{50} values. Thus, the difference in lethality in chick embryos inoculated with the virulent and avirulent types of gonococci is reproducible, and represents a real distinction between the two.

The viability of gonococci in the suspending medium (1% proteose peptone saline) decreased by about 40% in four hr., the time required for inoculation of the chick embryos for each set of experiments. Addition of 10% serum to the suspending medium did not improve viability. This decrease in viability would affect the precision of the LD_{50} determination but should have no effect in lethality differences based on these experiments since a) the decrease in viability for both the virulent (T1) and avirulent (T4) types is similar; b) the inoculation was done in a double blind setup; and c) the decrease in viability is within one log unit while the difference in lethality between the virulent and avirulent types is three log units.

Bumgarner and Finkelstein (1973) reported that death of chick embryos after intravenous inoculation of gonococci occurred mostly within 24 hr. In this study, while death of chick embryos inoculated with heat-killed organisms always took place within 24 hr., those inoculated with live organisms succumbed over a period of several days (see Appendix). As a result, LD_{50} values for live gonococci changed with

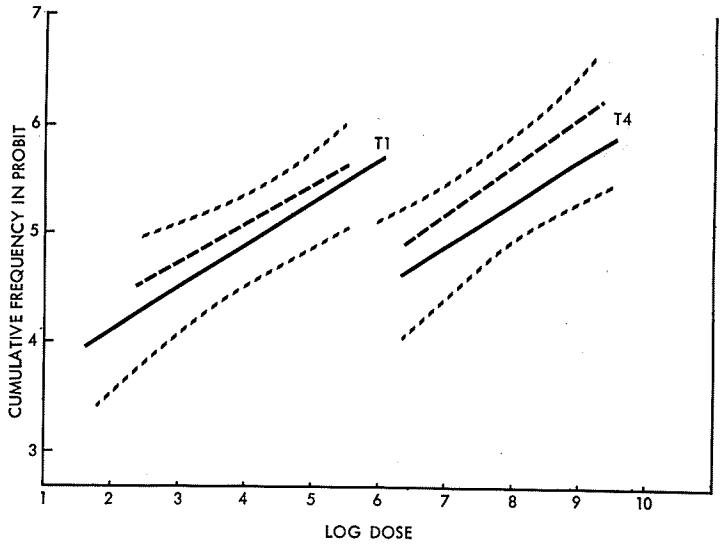


Figure 11. Comparison of two sets of LD_{50} determinations of types 1 and 4 of strain 3956 for 11-day chick embryos inoculated intravenously.

(——) represents one set of determinations of types 1 and 4. (---) represents another set performed at a different time.

respect to the length of the observation period (Table VIII).

The application of "latent period assay" gave a plot of median latent period versus log dose (Fig. 12). This graph confirms that T1 is significantly more virulent than T4. The fact that such a plot was obtained shows that the median latent period can be used as a bioassay for the relative virulence of gonococcal colony types. The slopes of the plots for T1 and T4 are significantly different.

Culture tests of blood samples from the dead embryos (Table IX) showed that from chick embryos inoculated with T4 organisms and dead within 24 hr., only T4 colonies were recovered. With chick embryos which succumbed during the 24-48 hr. period after inoculation, two of three showed predominantly T1 cells in their blood, since mainly T1 colonies were recovered upon culture of the blood. All of the chick embryos dying on day 4 showed the presence of only T1 cells in their blood. Culture tests of chick embryos inoculated with T1 cells showed no significant change in the colony type in their blood (Table X). The colonies recovered on culture remained predominantly T1.

Immunological Studies

a) With whole cells: Because of autoagglutinability, both live cell and formalin-treated whole cell suspensions were unsuitable for the tube agglutination test. Heat killed whole cells (120°C for 1 hr.) were satisfactory.

TABLE VIII

EFFECT OF LENGTH OF OBSERVATION PERIOD ON THE DETERMINATION OF LD_{50} OF STRAIN 3956

Colony Type	Time (Hr.) at which LD_{50} (CFU) was calculated				
	24	48	72	96	120
T1	-	9.10×10^{4}	6.10×10^{3}	2.81×10^{3}	2.81×10^{3}
T4	6.15×10^{8}	2.01×10^{7}	4.44×10^{6}	2.84×10^{5}	1.61×10^{5}

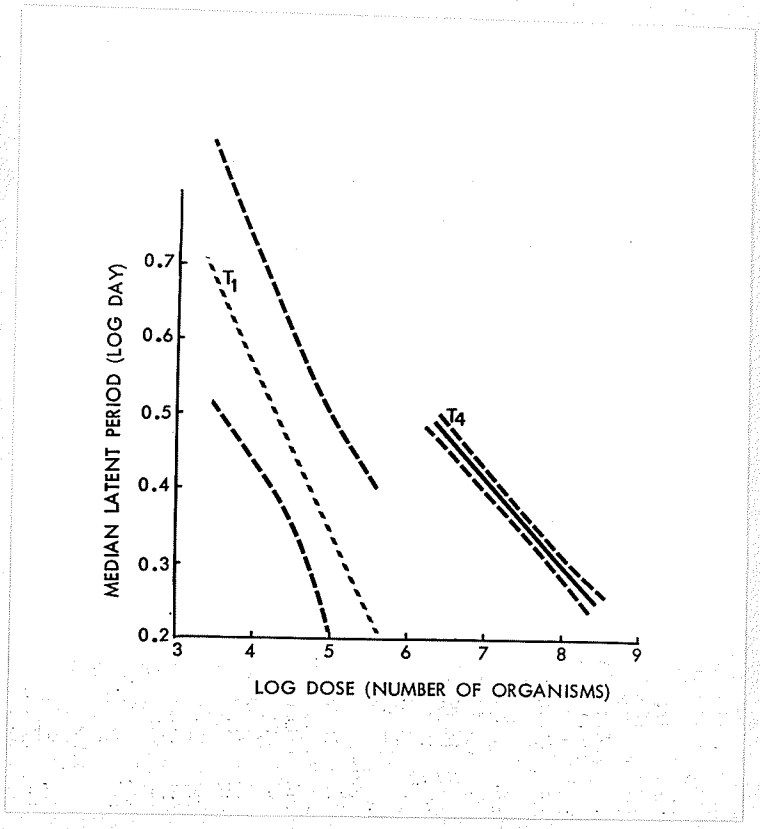


Figure 12. Relative virulence of types 1 and 4 (3956) for 11-day chick embryos inoculated intravenously: Relation of median latent period to log dose of inoculum.

Slope for T1 = -0.225. Slope for T4 = -0.116.

TABLE IX

FREQUENCIES OF CHICK EMBRYO LETHALITY WITH T4 (STRAIN 3956) AT SUCCESSIVE TIMES AND DILUTIONS

Time After Inoculation (Day)	SIZE OF INOCULUM (CFU)									
	2.31×10^9		2.31×10^8		2.31×10^7		2.31×10^6		Control	
	Deaths	CT *	Deaths	CT	Deaths	CT	Deaths	CT	Deaths	CT
1	27	T4	1	-	1	T4	2	T4	1	-**
2	0		14	T4	11	T1	11	T1	0	
3	0		6	T4	5	T1	1	T1 and T4	0	
4	1	T1	1	T1	4	T1	5	T1	1	-
5	0		1	ND***	0		1	ND	0	
CT of survivors 5 days after inoculation	-		-		-		T4		-	
Sum dead	28		23		21		20		2	
Number inoculated	29		29		28		29		28	

*CT = Culture test of one dead embryo. Only the predominant type (> 80%) is listed.
** - = No organisms recovered.
***ND = Not done.

TABLE X

FREQUENCIES OF CHICK EMBRYO LETHALITY WITH T1 (STRAIN 3956) AT SUCCESSIVE TIMES AND DILUTIONS

Time After Inoculation (Day)	SIZE OF INOCULUM (CFU)									
	3.34×10^5		3.34×10^4		3.34×10^3		3.34×10^2		Control	
	Deaths	CT*	Deaths	CT	Deaths	CT	Deaths	CT	Deaths	CT
1	1	T1	4	T1	8	T1	6	T1	1	-**
2	16	T1	7	T1	5	T4 and T1	3	T1	0	
3	4	T4 and T1	7	T1	0		1	T1	0	
4	4	T4	3	T1	1	T1	0		1	-
5	0		0		0		0		0	
CT of Survivors 5 days after inoculation	-		-		-		-		-	
Sum dead	25		21		14		10		2	
Number inoculated	29		29		30		29		28	

* CT = Culture test of one dead embryo. Only the predominant type (>80%) is listed.
** - = No organisms recovered.

In the tube agglutination test, no significant difference was detected between the various colony types of strain 3956 with antisera against T1 and T4 of the same strain. Strain F62 (T1 and T4) cross-reacted strongly with both antisera as shown in Table XI. No cross-reactivity was demonstrated with E. coli 320 cells. Cross-absorption of the antisera against T1 and T4 with T1 and T4 whole cells did not show any difference between T1 and T4, both of which were effective in absorbing out all the agglutinating activity of the two antisera (Table XII).

Similar conclusions could be drawn from the complement fixation tests (Tables XIII and XIV).

b) With LPS: The ultraviolet absorption spectra of LPS extracted from T1 and T4 cells are shown in Fig. 13. No distinct absorption peaks at 260 and 280 nm were found, showing that these preparations were essentially free of protein and nucleic acid. Infra-red absorption profiles of LPS preparations from T1 and T4 (Fig. 14) were typical of LPS from Gram-negative bacteria (Davies et al., 1958), indicating that carbohydrate and fatty acids are the main components. KDO assays gave a value of 3.68 and 4.46% (w/w) for T1 and T4 LPS preparations respectively.

Results of immunodiffusion testing with antisera to T1 and T4 whole cells and T1 and T4 LPS preparations are shown in Fig. 15. One major precipitation line was formed between the LPS and the homologous and heterologous antisera. No difference between T1 and T4 LPS was detected. The position

TABLE XI

TUBE AGGLUTINATION TITRES OBTAINED WITH HEAT-KILLED WHOLE CELLS OF STRAIN 3956

Antiserum against	Colony Type								
	T1	T1 (F62)	T1'	T2	T3	T4	T4 (F62)	T5	E. coli 320
T1	512	256	512	512	256	256	128	256	8
T4	256	128	512	256	256	256	128	256	8
Preimmune serum	< 8	-	-	-	-	< 8	-	-	-

TABLE XII

TUBE AGGLUTINATION TITRES OBTAINED AFTER ABSORPTION WITH HEAT-KILLED WHOLE CELL PREPARATIONS OF STRAIN 3956

Antiserum against	Absorbed with	T1	T4
T1	Unabsorbed	512	256
	T1	< 16	< 16
	T4	< 16	< 16
T4	Unabsorbed	256	256
	T1	< 16	< 16
	T4	< 16	< 16

TABLE XIII

COMPLEMENT FIXATION TITRES OBTAINED WITH HEAT-KILLED WHOLE CELLS OF STRAIN 3956

Antiserum against*	Colony Type								
	T1	T1 (F62)	T1'	T2	T3	T4	T4 (F62)	T5	E. coli 320
T1	128	128	128	128	128	256	256	256	16
T4	256	256	1024	256	256	1024	1024	1024	16
Preimmune serum	16	-	-	-	-	16	-	-	-

*All the sera were anti-complementary up to 1/16 dilution.

TABLE XIV

COMPLEMENT FIXATION TITRES AFTER ABSORPTION OF ANTISERA WITH HEAT-KILLED WHOLE CELLS OF STRAIN 3956

Antiserum against	Absorbed with	Strain 3956 Cells T1	Strain 3956 Cells T4
T1	unabsorbed	128	256
	T1	16	16
	T4	16	16
T4	unabsorbed	256	1024
	T1	16	16
	T4	16	16

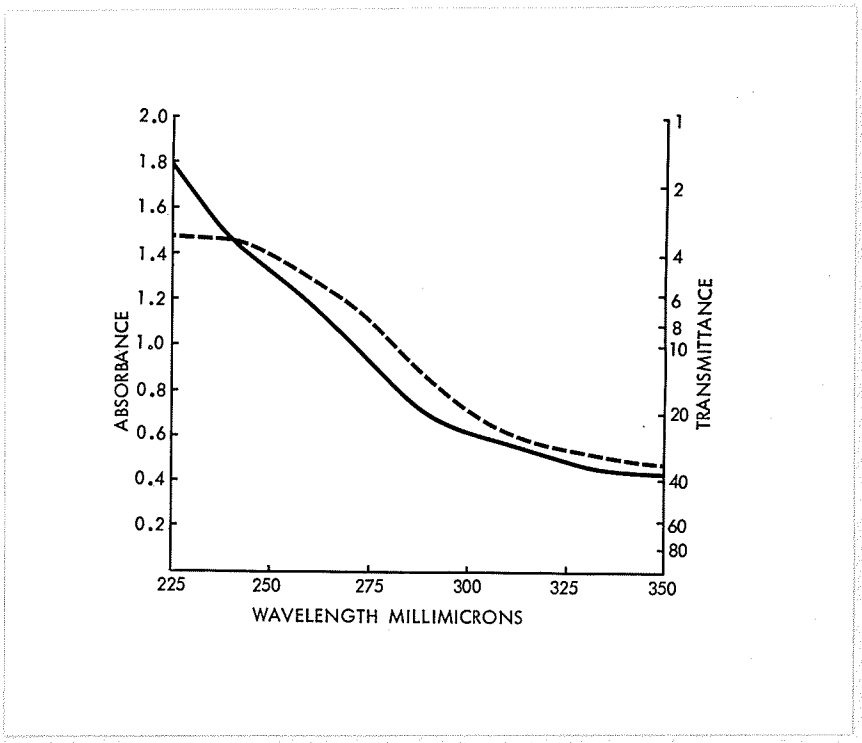


Figure 13. Ultraviolet absorption spectra of LPS extracted from types 1 and 4 (3956) cells.

The concentration of the LPS solutions was 1 mg/ml. (---) T4; (——) T1.

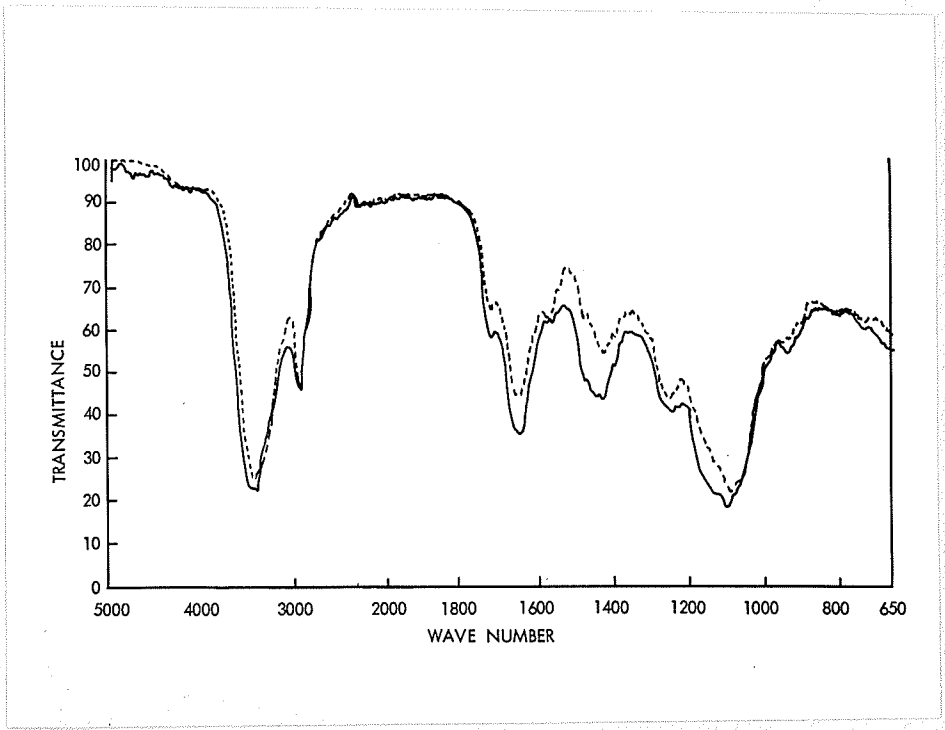


Figure 14. Infra-red absorption spectra of types 1 and 4 (3956) LPS.

(——) T1 LPS. (---) T4 LPS.

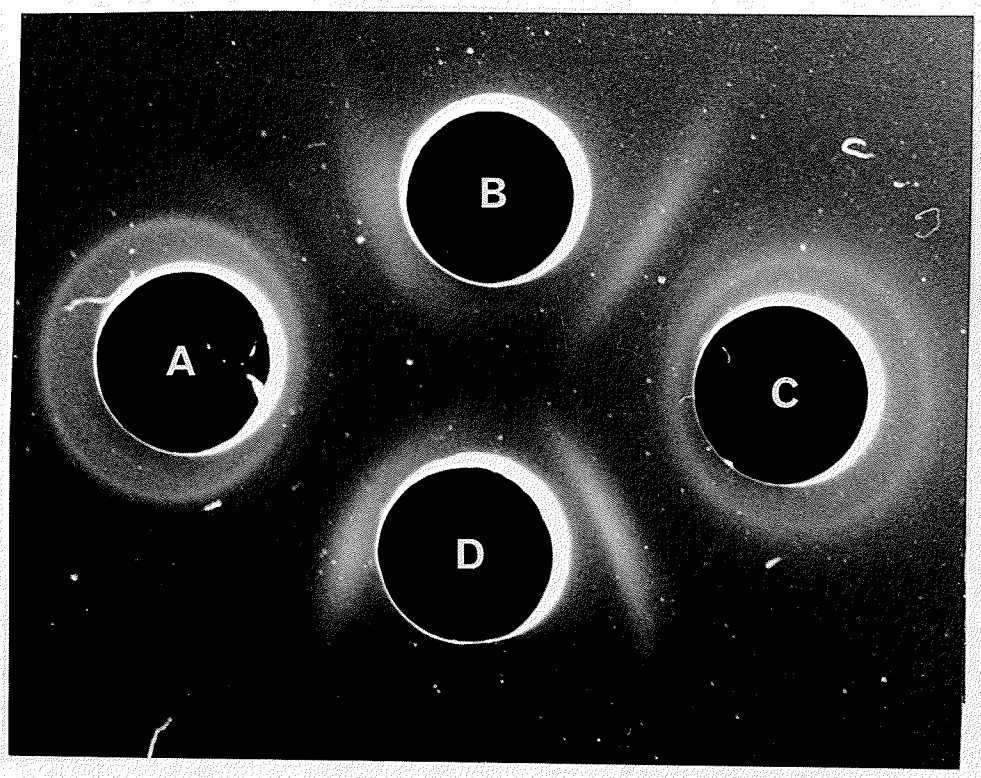


Figure 15. Immunodiffusion pattern; Antisera against T1 and T4 whole cells with T1 and T4 LPS.
A = antiserum against T1 whole cells.
B = T1 LPS.
C = antiserum against T4 whole cells.
D = T4 LPS.

and diffuse appearance of the precipitation line are characteristic of LPS, indicating that LPS is a high molecular weight substance.

Extracted LPS of Gram-negative bacteria has a high affinity for the surfaces of cells (Neter, 1956). This activity especially with erythrocyte membranes provides a more sensitive means of studying the immunological properties of LPS.

Gonococcal LPS (from T1 and T4) required either alkali or heat treatment before it would sensitize sheep erythrocytes (Table XV). Untreated LPS did not sensitize erythrocytes. Erythrocytes sensitized with alkali-treated LPS gave a higher titre than those coated with heat-treated LPS in the hemagglutination test, probably because sensitization with alkali-treated LPS gave a higher antigen concentration on the erythrocyte surface (Neter et al., 1956).

The effect of alkali treatment on T1 and T4 LPS was studied by infra-red scanning as shown in Fig. 16, and was identical for both T1 and T4 LPS. Two absorption peaks disappeared after such treatment; one at 1730 cm^{-1} and the other at 1250 cm^{-1}. These two peaks correspond to the absorption peaks of carboxyl C=O stretch and ester C-O stretch (Adams et al., 1969). The disappearance of these two peaks indicates that ester groups were destroyed by the alkali treatment. The general profile, however, remained the same which suggests that the chemical composition and structure were basically unchanged.

TABLE XV

EFFECT OF HEAT AND ALKALI TREATMENT ON HEMAGGLUTINATION TITRE

Treatment of T4 LPS*	Hemagglutination Titre with Anti-T4 Serum
None	< 8
100°C for 1 hour	64
0.02 N NaOH for 18 hours	256

*The sensitizing dose of LPS used was the same (1 mg/ml packed RBC). Residual RBC sensitizing activity of the supernatant of the sensitizing mixture was tested to assure maximal sensitization of the erythrocytes.

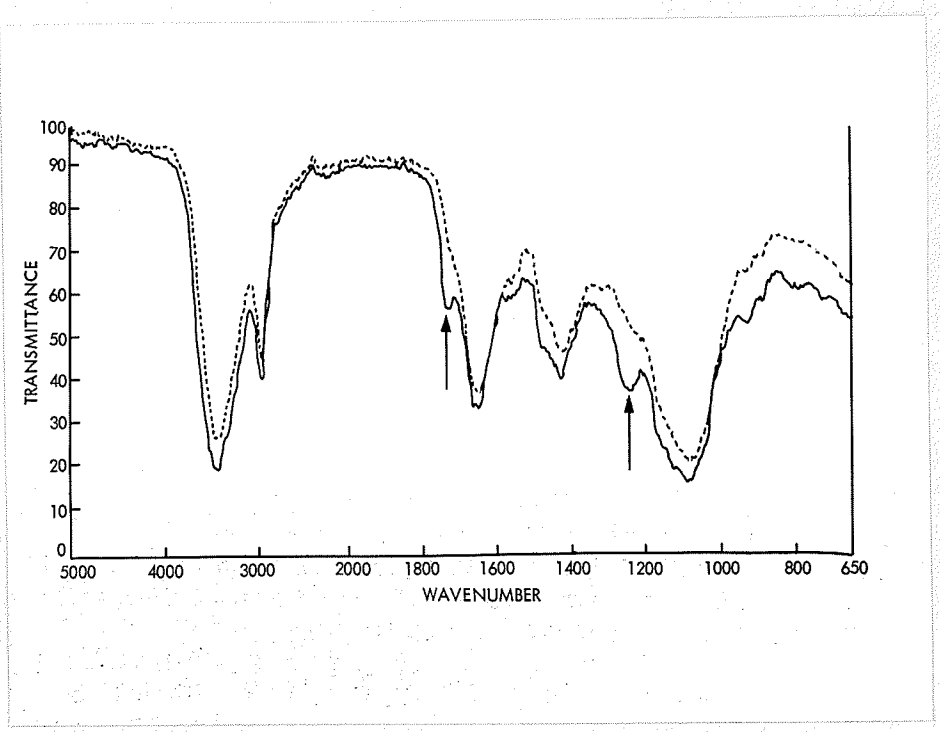


Figure 16. Infra-red absorption spectra which show the effect of alkali treatment on gonococcal LPS.

(——) untreated LPS. (---) alkali-treated LPS.

Glutaraldehyde fixation of sheep erythrocytes seemed to increase the "stickiness" of the cell surface so that even untreated LPS could be attached to it (Table XVI). The titres obtained, however, by use of sensitized fixed erythrocytes tended to be low and inconsistent. Therefore, subsequent studies were performed on unfixed erythrocytes sensitized with alkali-treated LPS. Time of sensitization of erythrocytes was one hour at 37°C, and increasing the sensitization time to two hours did not change the titre.

Erythrocytes sensitized with LPS were used in passive hemolysis and passive hemagglutination. Antisera used in tests involving sensitized red cells were always heat-inactivated and preabsorbed with unsensitized red cells to remove any non-specific activity. A comparison showed that the titres obtained from the two sets were consistent (Table XVII). The passive hemolysis test appeared to be more sensitive but less specific, because it provided a higher titre but also showed some activity with preimmune serum. Thus, the passive hemagglutination test was preferred. In both tests, erythrocytes sensitized with T1 LPS gave a higher titre with homologous antiserum. Aside from that, no difference was detected between T1 and T4 LPS.

Cross absorption data of antisera absorbed with sensitized erythrocytes are summarized in Table XVIII. Erythrocytes coated with T1 LPS absorbed all hemagglutinating activity from both antisera. While effective in absorbing hemagglutinating activity of antiserum against T4, erythrocytes

TABLE XVI

HEMAGGLUTINATION TITRE WITH GLUTARALDEHYDE-FIXED ERYTHROCYTES

Fixed RBC sensitized with LPS of	Antiserum against T1	Antiserum against T4
T1 (untreated)	8	8
(alkali-treated)	< 8	< 8
T4 (untreated)	< 8	128
(alkali-treated)	< 8	64

TABLE XVII

COMPARISON OF PASSIVE HEMOLYSIS AND PASSIVE HEMAGGLUTINATION TITRES OF GONOCOCCAL ANTISERA

Antiserum against	RBC sensitized with T1 LPS		RBC sensitized with T4 LPS	
	Hemolysis	HA	Hemolysis	HA
T1	2048	512	1024	256
T4	1024	256	1024	256
Preimmune serum	8	< 8	8	< 8

TABLE XVIII

HEMAGGLUTINATION AND TUBE AGGLUTINATION TITRES AFTER CROSS-ABSORPTION OF ANTISERA WITH SENSITIZED ERYTHROCYTES

Antiserum against	Absorbed with	RBC sensitized with T1 LPS		RBC sensitized with T4 LPS	
		HA*	TA**	HA	TA
T1	unabsorbed	256	512	128	512
	T1 - RBC	< 8	256	< 8	256
	T4 - RBC	64	256	<8	256
T4	unabsorbed	128	64	128	128
	T1 - RBC	< 8	32	<8	64
	T4 - RBC	<8	64	<8	64

*HA = Hemagglutination.
**TA = Tube agglutination.

sensitized with T4 LPS failed to absorb all activity of the antiserum against T1. However, tube agglutination titres of the two antisera were little affected even after repeated absorption with sensitized erythrocytes.

Hemagglutination titres after absorption of the antisera with formalin-treated whole cells of the various colony types are shown in Table XIX. No difference between the types was detected. A slight indication of some strain difference between F62 and 3956 was observed. Absorption with T1 (F62) whole cells did not remove all the activity of the two antisera.

In the hemagglutination inhibition test (Table XX), T1 LPS was effective in inhibiting the hemagglutinating activity of both antisera. On the other hand, four times the amount of T4 LPS was needed to inhibit the hemagglutinating activity of the antiserum against T1. Alkali-treated LPS preparations of T1 and T4 showed decreased antibody-neutralizing activity.

Inhibition of hemagglutination by sugars as shown in Table XXI indicated that all sugars tested failed to inhibit the activity of antiserum against T4. However, the activity of antiserum against T1 was affected by various sugars, notably lactose. The linkage seemed important in the specificity of this inhibition since both lactose and melibiose are composed of galactose and glucose, the only difference being that in lactose, the linkage between galactose and glucose is β1-4, and in melibiose it is α1-6. The structurally

TABLE XIX

HEMAGGLUTINATION TITRES AFTER ABSORPTION OF THE ANTISERA WITH FORMALIN-TREATED WHOLE CELLS OF STRAIN 3956

Absorbed with	RBC sensitized with T1 LPS		RBC sensitized with T4 LPS	
	Anti-T1 serum	Anti-T4 serum	Anti-T1 serum	Anti-T4 serum
unabsorbed	256	128	64	128
T1	< 8	< 8	< 8	< 8
T1*	16	8	16	8
T1'	< 8	8	< 8	8
T2	< 8	< 8	< 8	< 8
T3	< 8	< 8	< 8	< 8
T4	< 8	< 8	< 8	< 8
T4*	8	< 8	< 8	< 8
T5	8	< 8	< 8	8

*F62

TABLE XX

HEMAGGLUTINATION INHIBITION BY LPS PREPARATIONS OF STRAIN 3956

Antiserum against whole cells of*	RBC sensitized with LPS of	Amount (ug) necessary to inhibit HA			
		T1 LPS		T4 LPS	
		untreated	alkali-treated	untreated	alkali-treated
T1	T1	2x**	16x	8x	>32x
	T4	4x	32x	16x	>32x
T4	T1	8x	32x	16x	>32x
	T4	x	8x	4x	16x

*3 HA units of antisera was used.
**x = 0.195

TABLE XXI

INHIBITION OF HEMAGGLUTINATION BY SUGARS

Sugar Added	Titre with RBC sensitized with LPS of T1		T4	
	Anti-T1 serum	Anti-T4 serum	Anti-T1 serum	Anti-T4 serum
None	256	128	128	128
Glucose	256	64	128	128
Glucosamine	64	128	64	128
Galactose	64	128	32	128
Galactosamine	64	128	32	128
Levulose	64	128	128	128
Mannose	- *	-	-	-
Rhamnose	128	128	128	128
Cellibiose	128	128	64	128
Lactose	32	128	32	128
Melibiose	256	128	128	128
Maltose	64	128	128	128

*Mannose alone caused hemagglutination.

similar melibiose did not inhibit hemagglutination.

A summary of the effect of heat treatment and alkali treatment on gonococcal T1 and T4 LPS is presented in Table XXII. Alkali treatment of LPS increases its erythrocyte-sensitizing activity but decreases its antibody-neutralizing power as compared with untreated LPS.

TABLE XXII

SUMMARY OF EFFECT OF HEAT AND ALKALI TREATMENT ON LPS PREPARATIONS OF T1 AND T4 GONOCOCCI (STRAIN 3956)

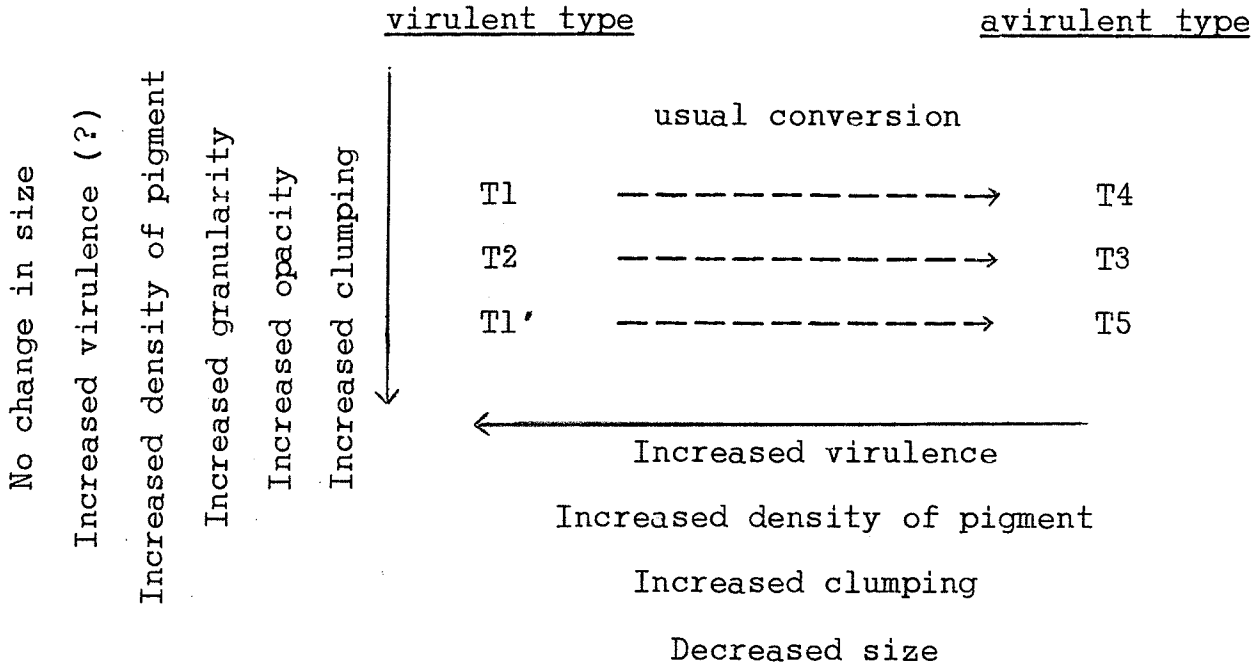
Treatment	Antibody neutralizing activity*	Erythrocyte modifying activity*
None	+++	-
100°C for 1 hr.	ND**	+
0.02 N NaOH for 18 hr.	+	+++

*- to +++ = various degree of activity.
**Not done.

DISCUSSION

The morphological features and physical properties of the colony types of N. gonorrhoeae observed in this study agree with the reports of Kellogg et al. (1968), Swanson et al. (1971), and Punsalang and Sawyer (1973). The physical properties of T1' and T5 have not been previously described. For colony types of strain 3956, sheep erythrocytes appeared to be a better indicator of hemagglutination than rabbit erythrocytes. Electron microscopy has confirmed the findings of other workers (Swanson et al., 1971; Jephcott et al., 1971): pili are absent on T3, T4 and T5, but are present on T1 and T2. Pili were also demonstrated on T1'. Pili are only associated with the virulent types (T1 and T2), and are generally regarded as a marker for virulence in gonococci. The fact that T1' also possesses pili as well as many properties of T1 and T2 suggests that it is a virulent type. This view is supported by the virulence study in chick embryos, as the LD_{50} of T1' is lower than those of the avirulent types.

The apparent close association between one particular colony type and another suggests that they are closely related genetically. The following diagram shows the interrelationships of the six colony types.



The ability of virulent types to agglutinate red cells has been attributed to the presence of pili (Punsalang and Sawyer, 1973). The observation that T5 which has no pili also agglutinates red cells indicates that other surface properties are involved.

In contrast to freeze-drying, which gives low recoveries of viable cells and a low degree of colony type stability with the virulent types of gonococci (Brookes and Heden, 1966; Caird, personal communication), the 'snap-freezing' method proves to be a suitable means of preservation for gonococci with respect to viability and colony type stability. Ward and Watt (1971) have reported that DMSO (6%) was not as good a protective agent as glycerol (8%), and higher DMSO concentrations are toxic to gonococci. The results of the present study showed no large difference between DMSO (10%) and

glycerol (8%). The discrepancy between the two studies is probably due to the difference of the media. Viable counts after freezing and thawing were higher than those before freezing. This may be a result of a combination of the following factors:

a) The breaking up of cell aggregates by freezing and thawing,

b) an uneven distribution of cells among the ampoules, and c) an improvement in precision of the viable count technique over that period of time.

No suitable liquid media have previously been found for the cultivation of virulent types of gonococci because of their instability. Jephcott (1972) has shown that the biphasic medium proposed by Gerhardt and Heden (1960) provided some degree of colony type stability of the virulent types. The ESP medium proves to be superior to the BP medium in terms of yield and colony type stability, both of which are dependent on the CO_2 tension imposed. At high CO_2 tensions (12% or 16%), the ESP medium is suitable for the cultivation of T1 (3956) and T1 and T2 of strain F62. Type 2 (3956) cells had a greater tendency to alter to other types than T1 cells and they multiplied very slowly in the ESP medium even when the CO_2 tension was as high as 16%. This indicates that T2 cells are more stringent than T1 cells in their nutrient and CO_2 requirements. Similar observations with colony types from other strains were reported by

Jephcott (1972). A further increase in the CO_2 tension may improve the colony type stability of T2 (3956) cells in the ESP medium, but high CO_2 tensions were also reported to be growth inhibitory (Jephcott, 1972).

Lethality experiments with 11-day chick embryos showed that in strain 3956 T1, T2 and T1' are virulent, while T3, T4 and T5 are not. Heat killed T1 and T4 cells had essentially the same degree of toxicity as live T4 cells. Thus, the difference in lethality of chick embryos inoculated with the virulent and avirulent types is not likely due to some intrinsic toxic factors present on the virulent types, but rather is a manifestation of the live gonococcal cells.

The LD_{50} value of T2 is much lower than that of T1. This may mean that T2 is more virulent than T1. However, despite such a big difference in the LD_{50} values, the difference may not be real since the two determinations were performed at different times.

The straight-line plots obtained in the median latent period assay showed that the time response could be used as a bioassay for the relative virulence of gonococci. The slopes for the plots of T1 and T4 are distinctly different; this may be a characteristic of the virulent and avirulent types. Therefore, the slope alone in such a plot may be used as an indicator of virulence.

The difference in the slopes of the T1 and T4 plots is an indication that the mechanisms of killing of chick

embryos by T1 and T4 are different. Culture tests showed that dead embryos teemed with gonococcal cells, and reversion from T4 to T1 in chick embryos with the progress of time was observed. It has been noted by Walsh et al. (1963) that the chick embryo acts as a selective medium for the virulent gonococcal cells, permitting their multiplication and suppressing growth of the avirulent ones. Bumgarner and Finkelstein (1973) showed that gonococcal cells of the virulent types were more resistant to clearing mechanisms of the blood stream and had a greater ability to multiply within the chick embryo. All this evidence suggests that the way the avirulent type (T4, in this case) kills the chick embryo is by the emergence of T1 cells by genetic mutation or selection. Being resistant to clearing mechanisms of the blood stream, the T1 cells survive and multiply, subsequently leading to the death of the embryo.

Cross absorption of the antisera with sensitized erythrocytes demonstrated that an extra antigenic determinant is present on T1 LPS which has all the antigenic determinants of T4 LPS. This conclusion is substantiated by the hemagglutination inhibition findings with T1 and T4 LPS.

The tube agglutination titres, however, were not significantly different after absorption of the antisera with sensitized erythrocytes. This means that the antibodies causing agglutination of gonococcal whole cells are directed to gonococcal surface determinants other than the LPS. This

would also explain the fact that tube agglutination tests failed to show any difference between T1 and T4 cells, despite the difference in their LPS moieties.

It is puzzling to find that even though there is a difference in the LPS of T1 and T4 cells, absorption of the two antisera with T1 and T4 whole cells failed to bring out this difference. One possible explanation is that since LPS is present on the cell surface as part of a complex, a greater degree of cross-reactivity may exist between the intact cell surfaces of the different colony types.

Sugar inhibition showed that hemagglutination of sensitized red cells with antiserum against T1 was sensitive to a number of sugars, such as galactose, glucosamine, galactosamine and lactose. Among them, lactose was the most effective one. Together with the fact that melibiose was totally ineffective, this suggests that galactosyl β1-4 glucose is part of the immuno-reactive component of T1 and T4 LPS (Maeland, 1969). Why then was the antiserum against T4 cells not sensitive to inhibition by sugars, including lactose?

This paradox could be explained as follows: on the intact cell surface of T4 cells, the carbohydrate moiety of the LPS is probably not very immunogenic and therefore antibodies against it made up only an insignificant fraction of the antiserum. This moiety on T4 LPS, though not very immunogenic, may be capable of combining with specific preformed antibodies. On the other hand, the capability of reacting with antibodies may be a result of extraction of the LPS such

that it is more exposed and therefore available for combination with antibodies.

Hexosamines, glucose and galactose are present in T1 as well as T4 LPS (Caird and Wiseman, personal communication). Therefore, it is plausible that a galactosyl β1-4 glucose unit may be a part of the gonococcal LPS structure.

Maeland (1969) proposed that gonococcal LPS is composed of two sets of antigenic determinants "a" and "b". The "a" set of determinants is polysaccharide in nature and the "b" set of determinants was thought to be a protein. Apicella and Allen (1973) demonstrated that the "b" set of determinants is a glycoprotein. The "a" set of determinants is made up of several antigenic determinants which determine serological specificity. Even with hot phenol-extracted LPS which contained a very small amount of protein, the determinant set "b" was also present (Maeland and Kristoffersen, 1971).

The following scheme, which can best explain the results of our serological tests, is proposed:

T1 LPS	T4 LPS
"b"	"b"
"a" = $a_1, \ldots a_{n+1}$	"a" = $a_1, \ldots a_n$

where determinant set "b" is common to T1 and T4 LPS, and determinant set "a" of T1 LPS contains n+1 "a" determinants. For type 4, n "a" determinants make up determinant set "a".

A great degree of cross-reactivity exists between T1 and T4 LPS, and there is an extra "a" determinant (denoted as

a_{n+1}) on T1 LPS. Both "a" and "b" sets of determinants are immunogenic on T1 cells, but on the intact cell surface of T4 cells, the "b" set of determinants is dominant. Other findings which support the presence of "a" set of determinants on T4 LPS are that "b" set of determinants alone is unable to hemadsorb and therefore incapable of sensitizing red cells, and that "b" set of determinants contains no heptose and KDO (Apicella and Allen, 1973). The recent findings of Perry and co-workers (1973) are consistent with our results. They found that T1 LPS was the S form whereas T4 LPS the typical R form.

Alkali treatment removes ester groups from gonococcal LPS as reported by Davies et al. (1958) and Ciznar and Shand (1970), working with LPS from other Gram-negative bacteria. We have shown that alkali-treated gonococcal LPS also has lower antibody-neutralizing activity. This means that the ester groups probably play some part in the immunospecificity of gonococcal LPS.

That an antigenic difference exists between LPS of T1 and T4 cells suggests that similar difference in the LPS moiety would be present between the other colony types. Further study to elucidate these differences may provide:

a) a scientific basis for the morphological typing of the organism,

b) a classification system for the various colony types, and

c) a possible correlation between the LPS structure and virulence in N. gonorrhoeae.

SUMMARY

1. Six colony types were identified and isolated from both strains of N. gonorrhoeae. They were designated T1, T1', T2, T3, T4 and T5, among which T1' has not previously been described in the literature. Some physical properties of the six colony types were investigated.

2. In addition to the possession of many properties of T1 and T2, T1' also has pili and was virulent in the chick embryo system. This suggests that T1' is more closely related to virulent types 1 and 2 than to avirulent types 3, 4 and 5.

3. The "snap-freezing" method was suitable for long-term preservation of virulent and avirulent types of gonococci, since a high degree of viability and colony type stability was obtained by this method. No large difference was found between glycerol and DMSO when these were used as a protective agent in the freezing process.

4. The colony type stability in BP and ESP media was dependent on CO_2 tension. Higher CO_2 tensions gave a greater degree of colony type stability. Both media can be used for the cultivation of T4 cells, as 100% colony type stability was obtained in either medium. However, the ESP medium provided a much better yield of all types than the BP medium, and only the ESP medium was satisfactory for cultivation of T1 (strain 3956), T1 (F62) and T2 (F62) cells.

5. Relative lethality in chick embryos showed that T1, T1',

and T2 had much lower LD_{50} values than T3, T4 and T5, suggesting that T1, T1' and T2 are the virulent types.

6. The median latent period assay can be used for the determination of virulence in N. gonorrhoeae. The killing mechanisms of virulent and avirulent types were discussed. The avirulent T4 reverted to T1 which survived, multiplied in and killed the chick embryo.

7. Hemagglutination tests with red cells coated with gonococcal LPS showed an extra antigenic determinant on T1 LPS which apparently had all the determinants of T4 LPS.

8. The majority of agglutinating antibodies present in the antisera were directed to gonococcal surface determinants other than the LPS.

9. Various sugars, notably lactose, inhibited the hemagglutination of red cells sensitized with T1 and T4 LPS in the presence of antiserum against T1. Based on these studies, it is suggested that galactosyl β1-4 glucose is a part of the gonococcal T1 and T4 LPS.

BIBLIOGRAPHY

Adams, G.A., T.G. Tornabene, and M. Yaguchi. 1969. Cell wall lipopolysaccharides from Neisseria catarrhalis. Can. J. Microbiol., 15:365.

Apicella, M.A., and J.C. Allen. 1973. Isolation and characterization of the β antigen of Neisseria gonorrhoeae. Infect. Immun., 7:315.

Arko, R.J. 1972. Neisseria gonorrhoeae: Experimental infection of laboratory animals. Science, 177:1200.

Atkin, E.E. 1925. The significance of serological types of gonococcus. Brit. J. Exp. Pathol., 6:235.

Bang, F. 1941. Experimental gonococcus infection of the chick embryo. J. Exp. Med., 74:387.

Beveridge, W.I.B., and F.M. Burnet. 1946. Medical Research Council Special Report Series No. 256: The Cultivation of Viruses and Rickettsiae in the Chick Embryo. Her Majesty's Stationery Office, London.

Bing, D.H., J.G.M. Weyand, and A.B. Stavitsky. 1967. Hemagglutination with aldehyde-fixed erythrocytes for assay of antigen and antibodies. Proc. Soc. Exp. Biol. Med., 124:1166.

Boor, A.K., and C.P. Miller. 1944. A carbohydrate-lipid fraction of gonococcus and meningococcus. J. Infect. Dis., 75:47.

Brookes, R., and C.-G. Heden. 1966. The preservation of Neisseria gonorrhoeae by controlled-rate freezing followed by storage in liquid nitrogen. Biotech. Bioeng., 7:315.

Brookes, R., and C.-G. Heden. 1967. Dense culture of Neisseria gonorrhoeae in liquid medium. Appl. Microbiol., 15:219.

Brown, W.J., and C.T. Lucas. 1973. Gonorrhoea in the chimpanzee: Serological testing. Brit. J. Vener. Dis., 49:441.

Brown, W.J., C.T. Lucas, and U.S.G. Kuhn. 1972. Gonorrhoea in the chimpanzee: Infection with laboratory passed gonococci and by natural transmission. Brit. J. Vener. Dis., 48:177.

Bryan, W.R. 1956. Biological studies on the Rous sarcoma virus. IV. Interpretation of tumor-response data involving one inoculation site per chicken. J. Nat. Cancer Inst., 16:843.

Buchanan, T.M., and E.C. Gotschlich. 1973. Studies on gonococcus infection. III. Correlation of gonococcus colony morphology with infectivity for the chick embryo. J. Exp. Med., 137:196.

Buchanan, T.M., J. Swanson, and E.C. Gotschlich. 1972. Antibody response to gonococcal pili in patients with gonorrhoea. J. Clin. Invest., 51:17a.

Buchanan, T.M., J. Swanson, K.K. Holmes, S.J. Kraus, and E.C. Gotschlich. 1973. Quantitative determination of antibody to gonococcal pili - changes in antibody levels with gonococcal infection. J. Clin. Invest., 52:2896.

Bumgarner, L.R., and R.A. Finkelstein. 1973. Pathogenesis and immunology of experimental gonococcal infection: Virulence of colony types of Neisseria gonorrhoeae for chick embryos. Infect. Immun., 8:919.

Carney, F.E., and D. Taylor-Robinson. 1973. Growth and effect of Neisseria gonorrhoeae in organ culture. Brit. J. Vener. Dis., 49:435.

Casper, W.A. 1937. The serological classification of gonococci by comparative agglutination. J. Bacteriol., 34: 353.

Catlin, B.W. 1973. Nutritional profiles of Neisseria gonorrhoeae, Neisseria meningitides and Neisseria lactamica in chemically defined media and the use of growth requirements for gonococcal typing. J. Infect. Dis., 128:178.

Chacko, C.W., and G.M. Nair. 1966. An immunologically active specific antigen from Neisseria gonorrhoeae grown in mebryonated chick eggs. Ann. Indian Acad. Med. Sci., 2:35.

Chanarin, I. 1954. An investigation of Neisseria gonorrhoeae by a red cell sensitization technique. J. Hyg., 54:425.

Ciznar, I., and J.W. Shands, Jr. 1970. Effect of alkali on the immunological reactivity of lipopolysaccharide from Salmonella typhimurium. Infect. Immun., 2:549.

Cohen, I.R. 1967. Natural and immune human antibodies reactive with antigens of virulent Neisseria gonorrhoeae: Immunoglobulin G, M and A. J. Bacteriol., 94:141.

Cohen, I.R., D.S. Kellogg, Jr., and L.C. Norins. 1969. Serum antibody response in experimental human gonorrhea. Immunoglobulins G, A, and M. Brit. J. Vener. Dis., 45: 325.

Cohen, I.R., and L.C. Norins. 1966. Natural human antibodies to Gram-negative bacteria: Immunoglobulin G, A, and M. Science, 152:1257.

Danielsson, D.G., J.D. Schmale, W.L. Peacock, Jr., and J.D. Thayer. 1969. Antigens of Neisseria gonorrhoeae: Characterization by gel-filtration, complement-fixation, and agar-gel diffusion of antigens of gonococcal protoplasm. J. Bacteriol., 97:1012.

Davies, D.A.L., M.J. Crumpton, I.A. Macpherson, and A.M. Hutchison. 1958. The absorption of bacterial polysaccharides by erythrocyte. Immunology, 2:157.

Davis, B.D., R. Dulbecco, H.N. Eisen, H.S. Ginsberg, and W.B. Wood, Jr. 1973. Microbiology. 2nd ed. Harper and Row Inc., New York. p. 742.

Deacon, W.E., W.L. Peacock, Jr., E.M. Freeman, and A. Harris. 1959. Identification of Neisseria gonorrhoeae by means of fluorescent antibodies. Proc. Soc. Exp. Biol. Med., 110:322.

Droge, W., V. Lehmann, O. Luderitz, and O. Westphal. 1970. Structural investigations on the 2-keto-3-deoxyoctonate region of lipopolysaccharide. Eur. J. Biochem., 14:175.

Eckert, E.A., D. Beard, and J.W. Beard. 1955. Dose-response relations in experimental transmission of avian erythromyeloblastic leukosis. III. Titration of virus. J. Nat. Cancer Inst., 14:1055.

Flynn, J. 1972. Resistance of rats to experimental infection with Neisseria gonorrhoeae despite attempts to alter cellular and humoral defences. Brit. J. Vener. Dis., 48:293.

Flynn, J., and S.A. Waitkins. 1973. Survival of Neisseria gonorrhoeae in an artificial subcutaneous cavity of the mouse. Brit. J. Vener. Dis., 49:432.

Frantz, I.D., Jr. 1942. Growth requirements of the meningococcus. J. Bacteriol., 43:757.

Gavrilescu, M., M. Lazar, I. Porojan, and T. Circiumarescu. 1966. Long-term cultivation of Neisseria gonorrhoeae in tissue culture. Brit. J. Vener. Dis., 42:171.

Gerhardt, P., and C.-G. Heden. 1960. Concentrated culture of gonococci in clear liquid medium. Proc. Soc. Exp. Biol. Med., 105:49.

Glynn, A.A., and M.E. Ward. 1970. Nature and heterogeneity of the antigens of Neisseria gonorrhoeae involved in the serum bactericidal reaction. Infect. Immun., 2:162.

Gould, R.G., L.W. Kane, and J.W. Mueller. 1944. On the growth requirements of Neisseria gonorrhoeae. J. Bacteriol., 47:287.

Griffin, P.J., and E. Racker. 1956. The carbon dioxide requirement of Neisseria gonorrhoeae. J. Bacteriol., 71:717.

Grimble, A.S., and M.B. McIllmurray. 1973. Cell mediated immune response in gonorrhoea. Brit. J. Vener. Dis., 49:446.

Hill, J.H. 1944. Experimental infection with Neisseria gonorrhoeae. II. Animal inoculations. Amer. J. Syph. Gonor. Vener. Dis., 28 334, 471.

Hunter, K.M., and I. McVeigh. 1970. Development of a chemically defined medium for growth of Neisseria gonorrhoeae. Antonie van Leeuwenhoek, 36:305.

Hurvell, B., P. Ahvonen, and E. Thal. 1971. Serological cross-reactions between different Brucella species and Yersinia enterocolitica. Agglutination and complement fixation. Acta Vet. Scand., 12:86.

Hurvell, B. 1972. Serological cross-reactions between different Brucella species and Yersinia enterocolitica. Immunodiffusion and immunoelectrophoresis. Acta Vet. Scand., 13:472.

Hutchinson, I. 1970. Typing the gonococcus. Brit. Med. J., 3:107.

James, A.N., R.D. Wende, and R.P. Williams. 1973. Variation in colonial morphology of Neisseria gonorrhoeae after growth on media containing antimicrobial agents. Appl. Microbiol., 26:248.

Jephcott, A.E. 1972. Preliminary study of colony type stability of Neisseria gonorrhoeae in liquid culture. Brit. J. Vener. Dis., 48:369.

Jephcott, A.E., and A. Reyn. 1971. Neisseria gonorrhoeae: Colony variation. I. Acta Pathol. Microbiol. Scand., 79B:609. 609.

Jephcott, A.E., A. Reyn, and A. Birch-Anderson. 1971. Demonstration of presumed appendages to cells from different colony types. Acta Pathol. Microbiol. Scand., 79B:437.

Kadis, S., G. Weinbaum, and S.J. Ajl (ed.). 1971. Microbial Toxin, Vol. 5. Academic Press Inc., New York.

Kearns, D.H., R.J. O'Reilly, L. Lee, and B.G. Welch. 1973. Secretory IgA antibodies in the urethral exudate of men with uncomplicated urethritis due to Neisseria gonorrhoeae. J. Infect. Dis., 127:99.

Kearns, D.H., G.B. Seibert, R.J. O'Reilly, L. Lee, and L. Logan. 1973. Paradox of the immune response to uncomplicated gonococcal urethritis. N. Eng. J. Med., 289:1170.

Kellogg, D.S., Jr., and W.E. Deacon. 1964. A new rapid immunofluorescent staining technique for identification of Treponema pallidum and Neisseria gonorrhoeae. Proc. Soc. Exp. Biol. Med., 115:968.

Kellogg, D.S., Jr., W.L. Peacock, Jr., W.E. Deacon, L. Brown, and C.I. Pirkle. 1963. Neisseria gonorrhoeae. I. Virulence genetically linked to clonal variation. J. Bacteriol., 85:1274.

Kellogg, D.S., Jr., I.R. Cohen, L.C. Norins, A.L. Schroeter, and G. Reising. 1968. Neisseria gonorrhoeae. II. Colonial variation and pathogenicity during 35 months in vitro. J. Bacteriol., 96:596.

Kenny, C.P., and B. Aris. 1969. The infection of tissue cells in culture with Neisseria gonorrhoeae. Can. J. Publ. Hlth., 60:34.

Kenny, C.P., F.E. Ashton, B.B. Diena, and L. Greenberg. 1967. A chemically defined protein-free liquid medium for the cultivation of some species of Neisseria. Bull. Wld. Hlth. Org., 37:569.

Kovalchik, M.T., and S.J. Kraus. 1972. Neisseria gonorrhoeae: Clonial morphology of rectal isolates. Appl. Microbiol., 23:986.

Kraus, S.J., G.H. Perkins, and R.C. Geller. 1970. Lymphocyte transformation in repeated gonococcal urethritis. Infect. Immun., 2:655.

Lankford, C.E. 1950. Chemically defined nutrient supplements for gonococcus culture media. Bacteriol. Proc., p. 40.

Lee, L., and J.D. Schmale. 1970. Identification of a gonococcal antigen important in the human immune response. Infect. Immun., 1:207.

Lindberg, A.A., and T. Holme. 1972. Evaluation of some extraction methods for the preparation of bacterial lipopolysaccharide for structural analysis. Acta Pathol. Microbiol. Scand., 80B:751.

Lucas, C.T., P. Chandler, Jr., J.E. Martin, Jr., and J.D. Schmale. 1971. Transfer of gonococcal urethritis from man to chimpanzee. An animal model for gonorrhea. J. Amer. Med. Ass., 216:1612.

Maeland, J.A. 1966. Antibodies in human sera against antigens in gonococci, demonstrated by a passive hemolysis test. Acta Pathol. Microbiol. Scand., 67:111.

Maeland, J.A. 1967. Serological properties of antisera to Neisseria gonorrhoeae antigens. Acta Pathol. Microbiol. Scand., 69:145.

Maeland, J.A. 1968. Antigenic properties of various preparations of Neisseria gonorrhoeae endotoxin. Acta Pathol. Microbiol. Scand., 73:413.

Maeland, J.A. 1969a. Antigenic determinants of aqueous ether extracted endotoxin from Neisseria gonorrhoeae. Acta Pathol. Microbiol. Scand., 76:475.

Maeland, J.A. 1969b. Immunochemical characterization of aqueous ether extracted endotoxin from Neisseria gonorrhoeae. Acta Pathol. Microbiol. Scand., 76:684.

Maeland, J.A. 1969c. Serological properties of aqueous ether extracted endotoxin from Neisseria gonorrhoeae. Acta Pathol. Microbiol. Scand., 77:495.

Maeland, J.A. 1969d. Serological cross-reactions of aqueous ether extracted endotoxin from Neisseria gonorrhoeae strains. Acta Pathol. Microbiol. Scand., 77:505.

Maeland, J.A., and T. Kristoffersen. 1971. Immunochemical investigations on Neisseria gonorrhoeae endotoxin. I. Characterization of phenol-water extracted endotoxin and comparison with aqueous ether preparations. Acta Pathol. Microbiol. Scand., 79B:226.

Maeland, J.A., T. Kristoffersen, and T. Hofstad. 1971. Immunochemical investigations on Neisseria gonorrhoeae endotoxin. 2. Serological multispecificity and other properties of phenol-water preparation. Acta Pathol. Microbiol. Scand., 79B:233.

Maeland, J.A., and B. Larsen. 1971. Human serum antibody reacting with endotoxin from Neisseria gonorrhoeae. Brit. J. Vener. Dis., 47:269.

Maeland, J.A., F. Wesenberg, and O. Tonder. 1973. Modified erythrocytes in indirect haemagglutination with Neisseria gonorrhoeae antigen. Brit. J. Vener. Dis., 49:256.

Martin, J.E., Jr., W.L. Peacock, Jr., G. Reising, D.S. Kellogg, Jr., E. Ribi, and J.D. Thayer. 1969. Preparation of cell walls and protoplasm of Neisseria gonorrhoeae with the Ribi cell fractionator. J. Bacteriol., 97:1009.

Miles, A.A., and S.S. Misra. 1938. The estimation of the bactericidal power of blood. J. Hyg., 38:732.

Miller, C.P., V. Moeller, and M. Bohnhoff. 1945. A method for production of a localized gonococcal infection in the rabbit's eye. Proc. Soc. Exp. Biol. Med., 58:143.

Mueller, J.H., and J. Hinton. 1941. A protein-free medium for primary isolation of the gonococcus and meningococcus. Proc. Soc. Exp. Biol. Med., 48:330.

Neter, E. 1956. Bacterial hemagglutination and hemolysis. Bacteriol. Rev., 20:166.

Neter, E., O. Westphal, O. Luderitz, E.A. Gorzynski, and E. Eichenberger. 1956. Studies of enterobacterial lipopolysaccharide: Effect of heat and chemicals on erythrocyte-modifying, antigenic, toxic and pyrogenic properties. J. Immunol., 76:377.

Osborn, M.J. 1963. Studies on the Gram-negative cell wall. 1. Evidence for the role of 2-keto-3-deoxyoctonate in the lipopolysaccharide of Salmonella typhimurium. Proc. Nat. Acad. Sci., 50:499.

Peacock, W.L., and J.D. Schmale. 1969. Toxic constituents of Neisseria gonorrhoeae. Nature, 221:760.

Perry, M.B., V. Daoust, B.B. Diena, F.E. Ashton, and R. Wallace. 1973. The lipopolysaccharides of Neisseria gonorrhoeae. Can. Publ. Hlth. Ass., p. 63.

Punsalang, A.P., Jr., and W.D. Sawyer. 1973. Role of pili in the virulence of Neisseria gonorrhoeae. Infect. Immun., 8:255.

Reising, G., D.S. Kellogg, Jr. 1965. Detection of gonococcal antibody. Proc. Soc. Exp. Biol. Med., 120:660.

Reising, G., J.D. Schmale, D.G. Danielsson, and J.D. Thayer. 1969. Reactivity of two selected antigens of Neisseria gonorrhoeae. Appl. Microbiol., 18:337.

Reyn, A., A.E. Jephcott, and H. Ravn. 1971. Neisseria gonorrhoeae. Colony variation II. Acta Pathol. Microbiol. Scand., 79B:435.

Rudbach, J.A. 1971. Molecular immunogenicity of bacterial lipopolysaccharide antigens: Establishing a quantitative system. J. Immunol., 106:993.

Schmale, J.D., D.G. Danielsson, J.F. Smith, L. Lee, and W.L. Peacock, Jr. 1969. Isolation of an antigen of Neisseria

gonorrhoeae involved in the human immune response to gonococcal infection. J. Bacteriol., 99:469.

Sparling, P.F. 1966. Genetic transformation of Neisseria gonorrhoeae to streptomycin resistance. J. Bacteriol., 92:1364.

Sparling, P.F., and A.R. Yobs. 1967. Colonial morphology of Neisseria gonorrhoeae isolated from males and females. J. Bacteriol., 93:513.

Stokinger, H.E., C.M. Carpenter, and J. Plack. 1944. Studies on the gonococcus. III. Quantitative agglutinative reactions of the Neisseria with special reference to Neisseria gonorrhoeae. J. Bacteriol., 47:149.

Swanson, J. 1972. Studies on gonococcal infection. II. Freeze-fracture, freeze-etch studies on gonococci. J. Exp. Med., 136:1258.

Swanson, J. 1973. Studies on gonococcal infection. IV. Pili: Their role in attachment of gonococcis to tissue culture cells. J. Exp. Med., 137:571.

Swanson, J., S. Kraus, and E.C. Gotschlick. 1971. Studies on gonococcus infection. 1. Pili and zones of adhesion: Their relation to gonococcal growth patterns. J. Exp. Med., 134:886.

Tauber, H., and W. Garson. 1957. Preparation and some properties of Neisseria gonorrhoeae endotoxin. Proc. Soc. Exp. Biol. Med., 95:669.

Tauber, H., and W. Garson. 1958. Effect of sonic energy, ultracentrifuge and phenol on Neisseria gonorrhoeae endotoxin. Proc. Soc. Exp. Biol. Med., 99:675.

Tauber, H., and W. Garson. 1959. Isolation of lipopolysaccharide endotoxin. J. Biol. Chem., 234:1391.

Tauber, H., and H. Russell. 1960. Amino compounds in lipopolysaccharides. J. Biol. Chem., 235:961.

Tauber, H., and H. Russell. 1961. Biochemistry of Neisseria gonorrhoeae endotoxin. Bull. Wld. Hlth. Org., 24:385.

Thayer, J.D., M.I. Perry, F.W. Field, and W. Garson. 1957. Failure of penicillin, chloramphenicol, erythromycin, and novobiocin to kill phagocytized gonococci in tissue culture. Antibiotics A, p. 513.

Thomas, D.W., J.C. Hill, and F.J. Tyeryar, Jr. 1973. Interaction of gonococci with phagocytic leukocytes from men and mice. Infect. Immun., 8:98.

Thongthai, C., and W.D. Sawyer. 1973. Studies on the virulence of Neisseria gonorrhoeae. 1. Relation of colonial morphology and resistance to phagocytosis by polymorphonuclear leukocytes. Infect. Immun., 7:373.

Tomiyama, T., J.Y. Homma, C. Abe, and M. Yoichi. 1973. Passive hemagglutination reaction using formalinized sheep erythrocytes treated with tanin and coated with protein moiety of the endotoxin (OEP) of Pseudomonas aeruginosa. Jap. J. Exp. Med., 43:185.

Waitkins, S.A., and J. Flynn. 1973. Intracellular growth and type variation of Neisseria gonorrhoeae in tissue cell-cultures. J. Med. Microbiol., 6:399.

Walsh, M.J., B.C. Brown, L. Brown, and C.I. Pirkle. 1963. Use of the chick embryo in maintaining and restoring virulence of Neisseria gonorrhoeae. J. Bacteriol., 86:478.

Ward, M.E., and A.A. Glynn. 1972. Human antibody response to lipopolysaccharide from Neisseria gonorrhoeae. J. Clin. Pathol., 25:56.

Ward, M.E., A.A. Glynn, and P.J. Watt. 1972. The fate of gonococci in polymorphonuclear leucocytes: An electron-microscopic study of the natural disease. Brit. J. Exp. Pathol., 53:289.

Ward, M.E., and P.J. Watt. 1971. The preservation of gonococci in liquid nitrogen. J. Clin. Pathol., 24:122.

Ward, M.E., P.J. Watt, and A.A. Glynn. 1970. Gonococci in urethral exudates possess a virulence factor lost on subculture. Nature, 227:382.

Watt, P.J. 1970. The fate of gonococci in polymorphonuclear leucocytes. J. Med. Microbiol., 3:501.

Watt, P.J., A.A. Glynn, and M.E. Ward. 1972. Maintenance of virulent gonococci in laboratory culture. Nature N. Biol., 236:186.

Watt, P.J., M.E. Ward, and A.A. Glynn. 1971. A comparison of serological tests for the diagnosis of gonorrhoea. Brit. J. Vener. Dis., 47:448.

Webb, M.J.W. 1973. A method for the rapid removal of sugars and salts from virus preparations on electron microscope grid. J. Microscope, 98:109.

Welton, J.P., H.E. Stokinger, and C.M. Carpenter. 1944. A chemically defined medium for the cultivation of the gonococcus. Science, 99:372.

Westphal, O., and K. Jann. 1965. In R.L. Whistler (ed.), Methods in Carbohydrate Chemistry, Vol. 5, Academic Press Inc., New York.

Westphal, O., O. Luderitz, and F. Bister. 1952. Uber die Extraktion von Bakterien mit Phenol/Wasser. Z. Naturforsch., 76:148.

Wilson, J.F. 1954. A serological study of Neisseria gonorrhoeae. J. Pathol. Bacteriol., 68:495.

APPENDIX

Experiment A. Incidence of chick embryo deaths after intravenous inoculation with gonococci.

Inoculum			Accumulative number dead		
Colony type	Dose (CFU/ml)	Number inoculated	Day 1	Day 2	Day 3
T1	5.36×10^{5}	11	1	6	9
	5.36×10^{4}	12	1	4	6
	5.36×10^{3}	11	1	2	2
	5.36×10^{2}	18	1	7	8
	5.36×10	11	1	1	1
T4	2.50×10^{9}	11	9	9	9
	2.50×10^{8}	11	7	7	7
	2.50×10^{7}	11	4	6	6
	2.50×10^{6}	11	0	4	4
T1 (Heat killed)	4.01×10^{8}	11	9	9	9
	4.01×10^{7}	12	6	6	6
	4.01×10^{6}	11	0	0	1
	4.01×10^{5}	11	0	0	0
T4 (Heat killed)	1.16×10^{8}	11	7	7	7
	1.16×10^{7}	11	11	11	11
	1.16×10^{6}	17	5	5	5
	1.16×10^{5}	11	1	1	1
Control		11	1	1	1

Experiment B. Incidence of chick embryo deaths after intravenous inoculation with gonococci.

Inoculum			Accumulative number dead		
Colony type	Dose (CFU/ml)	Number inoculated	Day 1	Day 2	Day 3
T1'	8.51×10^{6}	11	1	7	8
	8.51×10^{5}	11	4	7	8
	8.51×10^{4}	8	2	6	6
	8.51×10^{3}	11	5	8	9
	8.51×10^{2}	10	5	6	9
T2	1.00×10^{5}	10	7	10	10
	1.00×10^{4}	11	3	7	10
	1.00×10^{3}	10	5	6	6
	1.00×10^{2}	8	3	3	4
	1.00×10	11	5	6	7
T3	4.64×10^{8}	11	11	11	11
	4.64×10^{7}	11	2	5	6
	4.64×10^{6}	11	1	3	7
	4.64×10^{5}	11	1	3	4
	4.64×10^{4}	9	1	1	1
T5	1.68×10^{9}	10	10	10	10
	1.68×10^{8}	11	4	5	10
	1.68×10^{7}	11	0	0	2
	1.68×10^{6}	10	1	1	1
	1.68×10^{5}	10	2	2	4
Control		11	0	0	0