STUDIES RELATED TO THE DETECTION OF EXTRACHROMOSOMAL DNA IN SELECTED STRAINS OF NEISSERIA GONORRHOEAE

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ABSTRACT

STUDIES RELATED TO THE DETECTION OF EXTRACHROMOSOMAL DNA IN SELECTED STRAINS OF NEISSERIA GONORRHOEAE

By

Paul G. Engelkirk

Neisseria gonorrhoeae is the causative agent of gonorrhea, a venereal disease of epidemic proportions throughout the United States (62). Although N. gonorrhoeae has been cultivated in laboratories since 1882 (83), relatively little is known concerning the metabolic pathways, virulence or genetics of this microorganism.

Multiple antibiotic resistance and other phenomena associated with N. gonorrhoeae suggested that certain of its genetic markers might be plasmid-linked. Utilizing plasmid-curing and density gradient techniques, selected strains of N. gonorrhoeae were examined for evidence of plasmid DNA.

During the investigation techniques were developed for producing concentrated broth cultures of gonococci in relatively short time periods, for radioactive labeling of gonococcal nucleic acids, and for lysing the labeled cells with minimal damage to plasmid DNA.

The biphasic principle of Gerhardt and Hedén (20) was utilized to obtain broth cultures of the gonococci. A toxin-absorbing solid medium was overlaid with a clear, enriched, phosphate-buffered liquid medium which maintained the optimal pH for the organisms.

Gonococcal nucleic acids were labeled by transferring an aliquot of an early log phase biphasic flask system culture into prewarmed "labeling broth" containing ^{14}C-adenine. Sufficient incorporation was obtained to locate nucleic acid bands in the dye-buoyant density gradients.

The ^{14}C-labeled cells were lysed by a modification of the procedure of Clewell and Helinski (14). The procedure minimized enzymatic and physical damage of plasmid DNA, and yielded relatively clear and viscous lysates of N. gonorrhoeae and other microorganisms.

The results of the plasmid-curing experiments were contradictory, and thus provided no information regarding plasmids in multiple antibiotic resistant strains of N. gonorrhoeae.

The first known preliminary physical evidence of plasmids in N. gonorrhoeae was obtained by dye-buoyant density centrifugation of ^{14}C-labeled lysates. Plasmids may be peculiar to the strain utilized. Electron micrography revealed that certain colonial forms of this strain contained piliated cells.

A hypothesis was proposed to explain the correlation between plasmids and pili in this strain. When the episomic plasmid exists in its integrated state the host cell is capable of expressing pili. Plasmid-negative variants and cells harboring the plasmid in its autonomous state are incapable of expressing pili. The episomic plasmid hypothesis is similar to the hypotheses proposed to explain genetic competence in N. meningitidis (26, 27, 28, 29).

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INTRODUCTION

During the course of this investigation venereal disease was clearly out of control in the United States. Gonorrhea had reached epidemic proportions which some experts described as pandemic (62). In January 1972, the Surgeon General of the U.S. Public Health Service stated that gonorrhea was striking one person every 15 seconds (62).

One of the many factors which were contributing to the increased gonorrhea rate was the development of antibiotic resistance in the causative agent, Neisseria gonorrhoeae. The initial objective of this investigation was to develop techniques which would enable us to examine multiple antibiotic resistant strains of N. gonorrhoeae for evidence of R factors.

Immediately prior to and during the course of this investigation the initial reports of neisserial pili were published (25, 80, 94), and there was some speculation concerning correlations among gonococcal pili, colonial morphology and virulence of the organism (80). The rapid loss of a virulence factor (88) and rapid alterations in colonial morphology (32, 33) during in vitro cultivation suggested that the markers determining the virulence and/ or colonial

morphology of N. gonorrhoeae might be plasmid-linked. Another objective of this investigation was to examine piliated gonococci for evidence of extrachromosomal DNA.

It was hoped that evidence of extrachromosomal DNA in N. gonorrhoeae would contribute to the extremely limited body of knowledge concerning gonococcal genetics.

LITERATURE REVIEW

Neisseria gonorrhoeae

The term "gonorrhea", meaning "flow of seed or semen", was introduced by the celebrated Greek physician Claudius Galenus in the first century (83). In 1879, Albert Ludwig Siegmund Neisser, a German physician, identified the causative agent of gonorrhea, and called the organism "the gonococcus" because of its spherical shape (83). Today the organism is called *Neisseria gonorrhoeae*. *Neisseria gonorrhoeae* and *Neisseria meningitidis* are the only members of the family *Neisseriaceae* considered to be important pathogens for man.

The acceptable terms "gonococcus" (pl. "gonococci") and "gonococcal" (16) will be used herein in reference to *N. gonorrhoeae*.

Cellular morphology

Members of the genus *Neisseria* are non-motile, gram negative cocci, which may grow in pairs, tetrads or clusters of various sizes. *N. gonorrhoeae* and *N. meningitidis* usually form diplococci whose adjacent sides appear flattened when observed with the electron microscope, but may appear either

flattened or concave when Gram-stained specimens are observed with the light microscope. Individual cocci measure approximately 0.6 x 1.0 μm (7) and are longer on that side which is in contact with the other half of the diploid. Individual cocci have been described as "biscuit-shaped", "reniform", "kidney-like", "kidney-bean shaped" and "coffee-bean shaped".

Ultrastructure

Electron micrographs of N. gonorrhoeae thin sections have revealed a typical gram negative cell wall-membrane complex (17, 52, 80). The cell wall has been described as an undulating, convoluted or wrinkled triple-layered (triplex) or multi-layer structure (17, 34, 52). Beneath the cell wall are located, in sequence, an outer periplasmic space, a dense line, an inner periplasmic space, and one (17) or more (34) membrane-like structures. The wavy cell wall is in contact with the dense line at irregular intervals around the cell (17, 34). Mesosomes have been observed (17, 52), which are located in the cell periphery away from the division plane rather than near the plane of division as in gram positive organisms (17). Swanson et al. (80) reported that the nucleoid region and cytoplasm are very similar to those of N. meningitidis, and that the outer membrane and middle dense layer are morphologically identical to those of N. meningitidis (79).

Colonial morphology

Colonial variation in N. gonorrhoeae was reported as early as 1898 (90). Colonial morphology is influenced by such factors as 1) the composition of the solid medium employed, 2) the surface characteristics of the medium, including moisture content, 3) the age of the colonies, and 4) the technique of observation, especially the method of illumination (33). Descriptions and photographs of colonies are of little value unless conditions are specified and standardized.

Photographs, diagrams and lengthy descriptions of the four major gonococcus colony types appear in several references (24, 32, 33, 40, 63, 80). In general, under standardized conditions colony types 1 and 2 appear small (approximately 0.2 to 0.5 mm in diameter), round, convex, smooth, shiney and dark brown to black in color (32, 80). Colony types 3 and 4 appear larger (approximately 1.0 mm in diameter), round, flatter, more granular and lighter in color (32, 80). A fifth colony type (Type 5) was described by Jephcott and Reyn (24) and Reyn et al. (63).

Sparling and Yobs (75) and Jephcott and Reyn (24) demonstrated that most cultures obtained from patients with gonorrhea were composed predominantly of colony types 1 and 2. Kovalchik and Kraus (40) reported that rectal isolates of gonococci were predominantly colony types 1 and 2. Correlations between colonial type and virulence will be discussed under the section entitled Pathogenicity.

Unless they are selectively transferred, cultures of colony types 1 and 2 readily become cultures of colony types 3 and 4 (24, 32, 33). Quantitative data regarding conversions from one colony type to another (24, 32) are scanty at best. The studies by Kellogg et al. (32, 33) indicate that colony types 1 and 2 are genetically unstable and/or types 3 and 4 have a selective advantage over types 1 and 2 in vitro.

Kellogg et al. (32, 33) detected metabolic differences between different colony types of the same strain of N. gonorrhoeae. These differences will be discussed under the section entitled Metabolism and Nutritional Requirements.

Many bacteria are known to exhibit variation in colonial morphology. Differences in smooth and rough colonies of the same strain are frequently attributed to the presence or absence of some type of capsular material. Capsules have not been observed on cells from any of the four major colony types of N. gonorrhoeae, regardless of their source or culture medium (32).

To date, the only reported ultrastructural difference between cells from different colony types is the presence or absence of pili (80).

Neisserial pili

The first known report of pili (or fimbriae) in the genus Neisseria was published just prior to the start of this investigation (94). Pili were observed on N. catarrhalis,

N. perflava and N. subflava, but were not observed on the single strain of N. flava examined. The adhesive and coherent nature of the neisserial pili was revealed by hemagglutination tests and the use of a scanning electron microscope.

Jephcott et al. (25) published the first information pertaining to gonococcal "appendages". Although the authors found no direct connection between cell walls and the appendages, they felt that the appendages most closely resembled the pili of gram negative rods. The appendages were found in all suspensions of cells from colony types 1 and 2 but only in small numbers or entirely absent from suspensions of cells from colony types 3, 4 and 5.

Swanson et al. (80) published the first convincing electron micrographs of gonococcal pili. They observed pili on the surfaces of every specimen of colony types 1 and 2 gonococci but never on specimens of colony types 3 or 4. The number of pili per cell varied from a few (2 to 6) to many (25 to 50) which were observed in tangled masses surrounding small clumps of cells. The pili varied in length from 0.5 μm to 4 μm, which the authors speculated may have been due to fracturing of the pili or to different stages of growth or protrusion. Pilus diameter ranged from 80 to 85 Å. The pili exhibited a marked tendency toward lateral aggregation.

The presence or absence of pili may account for the differences in colonial morphology. Brinton (8) described a piliated form of Escherichia coli that grows on solid medium as a smaller, more opaque colony than the nonpiliated form. He hypothesized that the difference in colonial morphology may be due to the hydrophobic nature of the pili, which caused the piliated cells to adhere more tightly to each other than the nonpiliated cells. According to Swanson et al. (80), pili were observed only on those gonococci from the small colony types 1 and 2.

The only known reference to the biochemical composition of gonococcal pili is contained in a report by Jephcott et al. (25). They stated that impure suspensions of gonococcal "fibrils" contained some carbohydrate, but protein and lipid were not major components. This is in contrast to Type I pili of E. coli which are pure proteins (8).

The function of gonococcal pili is unknown. Possible functions of pili include transport of metabolites, transport of nucleic acid into or out of the cell, transport of bacteriocins, or adhesion to surfaces (8). If gonococcal pili served to transport materials including DNA into the cells, this could perhaps explain the findings of Sparling (74) that colony types 1 and 2 gave transformation frequencies which were 2×10^4 times greater than those of colony types 3 and 4. Correlations between gonococcal pili and virulence will be discussed under the section entitled Pathogenicity.

Fastidious nature

N. gonorrhoeae is considered to be a fastidious organism, which cannot survive once removed from the host unless a number of conditions are met. *In vitro* cultivation requires the use of enriched media, reduced oxygen tension and a moist atmosphere. The organism has very narrow optimal temperature (35 to 36 C) and pH (7.2 to 7.6) ranges (83).

The fastidious nature of *N. gonorrhoeae* may be due more to sensitivity to inhibitors than to complexity of nutritional requirements (67). The organism is susceptible to toxic substances such as fatty acids (43), polyamines (66) and traces of detergents and heavy metal ions (15).

N. gonorrhoeae is microaerophilic, requiring a reduced oxygen tension for *in vitro* cultivation (15). This requirement is normally accomplished by incubation in candle jars or CO_2 incubators in an atmosphere of 3 to 10% CO_2. Candle jars provide an atmosphere of approximately 3% CO_2 (2). *N. gonorrhoeae* cells do not seem to have any special requirement for CO_2 but a normal oxygen tension is toxic during their initial stages of growth (15).

Metabolism and Nutritional Requirements

Although *N. gonorrhoeae* was first cultivated *in vitro* in 1882 (83), relatively little is known regarding the

metabolic pathways and nutritional requirements of this organism. Lankford (41) reported that 23% of 1042 gonococcal isolates required the addition of glutamine to a proteose-peptone hemoglobin agar, and 0.8% required cocarboxylase (the coenzyme, thiamine pyrophosphate). Tonhazy and Pelczar (86) reported that the strain of N. gonorrhoeae with which they were working could oxidize α-ketoglutarate, succinate, fumarate, malate, oxaloacetate and pyruvate, but could not oxidize citrate or acetate. Of all amino acids tested, only L- and D-glutamic acid were oxidized at an appreciable rate. The observation that N. gonorrhoeae is incapable of oxidizing citrate was also reported by Hill (21).

Although there are no known reports concerning utilization of exogenous nitrogenous bases by N. gonorrhoeae, Kingsbury and Duncan (38) reported that N. meningitidis does not require an exogenous supply of purine and pyrimidine bases. Their studies indicated that adenine was by far the most actively utilized compound when a number of deoxyribonucleosides, ribonucleosides and free bases were tested. Guanine and uracil were the only other compounds which were incorporated in significant amounts. Jyssum and Jyssum (31) confirmed the findings of Kingsbury and Duncan (38) that N. meningitidis takes up thymine, thymidine and TMP to a very limited extent. Jyssum (30) later reported that N. meningitidis lacks the enzyme systems that are known to mediate a specific incorporation of thymine or thymidine into DNA.

Gonococci produce acid but no gas from glucose (7). During growth in glucose-containing medium, the production of acid may be slight, and alkaline products of peptone degradation may neutralize the acid (13). Gonococci do not produce acid from fructose, lactose, maltose, mannitol or sucrose (13).

Kellogg et al. (33) reported that type 1 colonies produced acid from glucose at a slower rate than colony types 2, 3 and 4. Such findings correlated well with observations reported many years earlier by Morton and Shoemaker (51). In a later report, Kellogg et al. (32) stated that type 2 colonies were capable of growing throughout agar-medium shake tubes, whereas colony types 1, 3 and 4 were not.

Enzyme production

Members of the genus Neisseria produce the enzyme "oxidase", which produces colored compounds from aromatic amines in the presence of air (57). This reaction on N,N-Dimethyl (or Tetramethyl)-p-phenylenediamine monohydrochloride forms the basis of the "oxidase test", which is routinely performed during identification of Neisseria species (81, 82). "Oxidase" is probably cytochrome oxidase, the terminal cytochrome of the respiratory chain (42, 76).

Members of the genus Neisseria produce abundant quantities of the enzyme, catalase (7), which catalyzes the conversion of hydrogen peroxide to water and oxygen. Accumulations of hydrogen peroxide are bactericidal.

Pathogenicity

Although gonorrhea and other manifestations of gonococcal disease have plagued man for centuries, relatively little is known concerning the virulence of N. gonorrhoeae.

There exists no evidence for exotoxin production by N. gonorrhoeae. The organism produces an endotoxin composed of lipid, carbohydrate and protein (44). Protein constituted the major constituent, and glucose, galactose, glucosamine and heptose were detected (44). The role of this endotoxin in pathogenesis has not been defined.

N. gonorrhoeae demonstrates some degree of tissue specificity (15). The organism is apparently incapable of penetrating stratified squamous epithelium, but capable of penetrating intercellular spaces of stratified columnar epithelium, such as is found in mucous membranes (83).

Gonococci do not produce hemolysins, coagulases or fibrinolysins (32), and information concerning DNAse production is contradictory (33, 74).

Gonococci are classified as pyogenic cocci (15), and a purulent discharge is associated with gonococcal urethritis in the male. The discharge contains many

polymorphonuclear leukocytes ("pus cells"). Classification of N. gonorrhoeae as a facultative intracellular parasite has been apparently based upon the frequent observation of morphologically-intact diplococci within the "pus cells". A recent investigation by Watt (92) suggests that the virulence of gonococci cannot be attributed simply to survival of gonococci in polymorphonuclear leukocytes.

Ward et al. (88) have reported that in vivo gonococci possess a protective factor which enables them to resist killing by natural antibodies and complement. The factor, which they suggest may be a surface antigen, is quickly lost during in vitro cultivation.

Thongthai and Sawyer (85) reported that resistance of gonococci to phagocytosis is correlated with colonial morphology. Cells from colony types 3 and 4 were more susceptible to phagocytosis than cells from colony type 1. Sawyer thinks that the virulence of gonococci may be attributed solely to their ability to resist phagocytosis (Personal communication).

Since 1898, when colonial variation in N. gonorrhoeae was first reported (90), many investigators have speculated about a correlation between colonial morphology and virulence of the organism (1, 11, 12, 22). It was not until 1963, however, that evidence for such a correlation was published (33). Kellogg et al. (32) reported that cells from colony types 1 and 2 were capable of causing gonococcal

urethritis in human male volunteers; whereas, cells from colony types 3 and 4 were incapable of causing disease. They concluded that the genetic bases of colonial morphology and virulence were closely related in the gonococcal genome (32).

Swanson et al. (80) stated that the consistent presence of pili on virulent colony types 1 and 2 and the absence of pili on avirulent colony types 3 and 4 suggests a relationship between the pili and the pathogenic potential of the gonococci. The authors speculated that the pili may serve an antiphagocytic function or that the adhesive nature of the pili may enable the cells to adhere to surfaces which are constantly flushed or cleansed, such as the eye or the urinary tract.

Antibiotic resistance

Treatment of gonorrhea was for the most part unsuccessful until the advent of the sulfonamide drugs in the late 1930's. Due to the widespread use of these agents during World War II, most strains of gonococcus became sulfonamide resistant. Introduction of penicillin treatment in 1943 restored control over the sulfonamide resistant strains, and penicillin remains the antibiotic of choice in the treatment of gonococcal disease (15).

During the past two decades, however, numerous investigations have revealed that N. gonorrhoeae has become

increasingly more resistant to penicillin (47, 48, 65, 84). One study (47) revealed that 99.4% of the gonococcal isolates collected during the period 1945 to 1954 were susceptible to 0.05 units (approximately 0.03 µg) of penicillin per ml, but only 35% of the isolates collected during the period 1968-1969 were susceptible to that level. Fourteen per cent of the isolates required more than 0.5 units (approximately 0.3 µg)/ml for inhibition. Strains requiring concentrations as high as 3.5 units (approximately 2.0 µg)/ml had been isolated.

To further compound the problem of treatment of gonococcal disease, many strains have become resistant to more than one antibiotic. Thayer et al. (84) reported that strains resistant to 0.2 units or more of penicillin/ml are almost always streptomycin resistant. Martin et al. (48) reported that an increase in penicillin resistance was accompanied by a decrease in cephalothin and cephaloridine susceptibility. Increased resistance of gonococci to tetracycline (65), spectinomycin (58), and rifampicin (45) has been reported also.

Gonococcal DNA

DNA from the genus Neisseria is characterized by equimolar concentrations of the bases guanine, adenine, cytosine and thymine. The following data were reported by Belozersky and Spirin (5):

	Base Proportions (moles %)			
	guanine	adenine	cytosine	thymine
N. gonorrhoeae	25.2	25.3	24.4	25.1
N. meningitidis	25.5	24.6	25.0	24.9

The mole % guanine + cytosine (i.e., %GC) of N. gonorrhoeae is thus 49.6.

Kingsbury (37) calculated that there are approximately 1.5×10^6 nucleotide pairs in N. gonorrhoeae DNA. Assuming an average molecular weight of 660 daltons for each nucleotide pair (91), the molecular weight of the N. gonorrhoeae chromosome is approximately 9.9×10^8 daltons (i.e., $660 \times 1.5 \times 10^6$).

Estimates of the molecular weight of the E. coli chromosome range from 2.5×10^9 daltons (91) to 2.97×10^9 daltons calculated from the data of Kingsbury (37). The N. gonorrhoeae chromosome is therefore only 33.3 to 39.6% as large as the chromosome of E. coli and may be capable of coding for only approximately one-third of the number of proteins coded for by E. coli.

Extrachromosomal DNA

The term "extrachromosomal DNA" is used in reference to deoxyribonucleic acid molecules within a bacterial cell which are physically separate from the bacterial chromosome.

The term "plasmid" is used in reference to an independent (i.e., extrachromosomal) replicon found within a bacterial cell (64). All plasmids thus far identified are independent replicons and, therefore, most likely consist of double-stranded DNA molecules (64). Plasmids normally contain only a small amount of genetic material in comparison to the bacterial chromosome and are not essential for the survival of the cell (64).

The term "episomal plasmid" is used in reference to a plasmid that is capable of reversible transition between chromosomal and extrachromosomal states (64).

Many of the plasmids detected and studied carry antibiotic resistance markers (64). Other markers in E. coli which have been demonstrated to be plasmid-linked are colicin production (4), K88 antigen production (56), enterotoxin production (72), and hemolysin production (71). F-factors, or sex factors, represent another class of plasmids. Some bacteriophage also satisfy the plasmid criteria.

R factors

In 1960 it was reported that multiple antibiotic resistance could be transferred from Shigella flexneri to E. coli, and that the property of transmissible antibiotic resistance existed extrachromosomally and replicated autonomously (49). Shortly thereafter, the term "R (resistance) factor" was adopted for the property of transmissible antibiotic resistance (49).

R factors are episomic plasmids capable of transfer by conjugation among most species of the family Enterobacteriaceae, and from various enteric organisms to Vibrio cholerae, Yersinia pestis and Yersinia pseudotuberculosis (49).

R factors have been reported to carry resistance markers for tetracycline, chloramphenicol, streptomycin, sulfonamides, kanamycin, neomycin and penicillin in addition to other antibiotics and inorganic ions such as Hg^{++}, Co^{++} and Ni^{++} (53). R factors are of medical importance because multiple antibiotic resistance markers may be transferred en bloc from non-pathogenic organisms of the normal intestinal flora to invading pathogens.

Curing of plasmids

When a plasmid is selectively inactivated or inhibited in replication by physical or chemical agents, leading to a high frequency of plasmid-negative variants, the plasmid is said to be "cured" (53, 64).

A "curing agent", therefore, is any physical or chemical agent that interferes with plasmid replication or distribution without having an equivalent effect upon the host chromosome (64).

Novick (53) established the following criteria as acceptable evidence for curing:

1. The frequency of plasmid-negative variants is increased in the presence of the curing agent.

2. Under the curing conditions the plasmid-negative variants grow at the same rate as their parent strain.

3. The plasmid-negative variants do not revert.

Ethidium bromide (6), acridine dyes (23) and sodium dodecyl sulfate (73) have been utilized as chemical curing agents with varying degrees of success. Ethidium bromide and the acridine dyes are known to intercalate with DNA, thus interfering with DNA replication (6). Plasmid replication is apparently more sensitive to this phenomenon than chromosomal replication. Sodium dodecyl sulfate (SDS) is known to cause disruption of biological membranes (73). Sonstein and Baldwin (73) speculated that SDS curing of penicillinase plasmids in Staphylococcus aureus may have been due to disruption of the membrane sites of plasmid attachment.

Physical evidence of plasmids

Novick (53) has stated that the demonstration of a correlation between a given phenotype and the presence of a specific circular DNA molecule will strengthen greatly any of the more circumstantial kinds of evidence for plasmid-linked inheritance. He has further stated that if a given gene is plasmid-linked, plasmid DNA should be demonstrable in those cells carrying the gene and absent from those cells lacking it.

Genetic experiments and examination of plasmid DNA with the electron microscope have demonstrated that plasmids exist as circular molecules for at least some stage of their existence (64).

There are a number of techniques utilized to separate plasmid DNA from host chromosomal DNA having the same %GC (and buoyant density). Many of these techniques are based upon physicochemical differences between circular, covalently closed DNA molecules and linear DNA molecules (61). Closed circular DNA is more resistant to denaturation, has a greater sedimentation velocity in neutral and alkaline solutions, has a greater buoyant density in alkaline solution, and binds fewer molecules of ethidium bromide (61).

Zonal (velocity type) Density Gradient Centrifugation (15, 42)

Separation of molecules having different sedimentation coefficients can be achieved by layering them onto a preformed linear sucrose gradient and subjecting them to high speed centrifugation. The various components form bands at various levels in the gradient, depending upon particle size, shape and density. Good separations are achieved in relatively short periods of time because the sucrose concentration gradient is preformed. Fractions are collected as drops by puncturing the bottom of the tube.

During fraction collection the bands maintain their relative positions because the density gradient prevents convection and mixing.

Sedimentation coefficients of nucleic acids have very low values when expressed in absolute values. For this reason values are usually expressed in Svedberg (S) units. The sedimentation coefficient of a given DNA molecule can be calculated following cosedimentation in a sucrose density gradient with a DNA molecule of known sedimentation coefficient. For example, Olsen and Schoenhard (55) utilized a 23S $ColE_1$ molecule to calculate the sedimentation coefficients of the PO-1 and PO-2 plasmids of S. pullorum MS53.

Linear DNA is totally denatured in 0.3 M NaOH at 20 to 25 C (19). Circular, covalently closed DNA molecules are more resistant to alkali denaturation than are linear DNA molecules (61). Plasmids, therefore, sediment faster in alkaline sucrose gradients than do linear or nicked circular DNA molecules (61).

Equilibrium Density Gradient Centrifugation (15, 42)

Equilibrium centrifugation in cesium chloride solutions is frequently utilized to determine the buoyant density of DNA molecules or to separate DNA molecules of different buoyant density. The sample is mixed in a centrifuge tube with the high molecular weight salt, cesium chloride. The tube is centrifuged for a relatively long period of time

during which a stable concentration gradient of cesium chloride is formed. DNA molecules that are present will concentrate into a stable band at that position in the tube at which their buoyant density is exactly equal to the density of the cesium chloride solution. The actual density at any point in the tube can be calculated.

The location of the band is independent of the size and shape of the particles, and components are separated solely on the basis of differences in density. The buoyant density of nucleic acids at neutral pH depends upon the type of sugar present (i.e., ribose or deoxyribose), the strandedness of the molecules, and the base ratio. In general, RNA is more dense than DNA, single-stranded DNA is more dense than double-stranded DNA, and the density of double-stranded DNA increases linearly with its proportion of guanine plus cytosine.

Fractions are collected as drops from the bottom of the tube, as in zonal (velocity type) density gradient centrifugation.

Addition of ethidium bromide to the cesium chloride mixture permits the separation of circular, covalently closed DNA molecules from linear DNA molecules having the same or similar GC ratio (61). Bazaral and Helinski (4) described a rapid technique for the isolation of closed, double-stranded DNA from bacteria based upon the dye-buoyant density procedure of Radloff et al. (61). Separation of DNA

molecules of different configurations is accomplished as follows (61):

1. Molecules of the dye, ethidium bromide apparently intercalate between adjacent base pairs of the double helical DNA molecule.

2. Closed, circular duplex DNA molecules bind fewer molecules of ethidium bromide than do linear or nicked circular DNA molecules.

3. The buoyant density of DNA-ethidium bromide complexes is inversely proportional to the number of dye molecules bound.

4. At saturation, the buoyant density of the closed circular DNA-ethidium bromide complex is greater than that of the linear or nicked circular DNA-ethidium bromide complex.

5. Closed circular DNA will band lower in the density gradient tube than will linear or nicked circular DNA.

EDTA is added to the mixture to chelate Mg^{++} ions. Mg^{++} ions markedly inhibit interaction of ethidium bromide molecules with DNA molecules (89). Mg^{++} ions also serve as cofactors for some types of DNAse.

Extrachromosomal DNA in the Genus Neisseria

Jyssum and Lie (29) and Jyssum (26) speculated that the genetic determinant for competence in N. meningitidis may be located on an episomic plasmid. Their speculation

was based upon the high frequency with which competence is lost and an apparent lack of reversion to genetic competence. Jyssum and Jyssum (28) obtained evidence that a competent variant of one strain contained more DNA than an incompetent variant of the same strain. Although this observation was offered as additional evidence for an episomic plasmid, they were unable to demonstrate significant differences between cesium chloride gradients of these strains. In a more recent publication, Jyssum (27) reported that ethidium bromide and acridine orange were successfully utilized to eliminate competence in two strains of N. meningitidis.

Kingsbury (36) was the first to report evidence for bacteriocin production in the genus Neisseria. He speculated that the bacteriocins produced by N. meningitidis ("meningocins") may be episomal plasmids based upon the inducible nature of one of the bacteriocins by ultraviolet irradiation and by mitomycin C.

Bacteriocins from N. gonorrhoeae ("gonocins") have recently been reported by Flynn and McEntegart (18), but the authors were unable to induce bacteriocin production by exposure to ultraviolet light or the addition of mitomycin C to liquid cultures.

There have been several reports concerning bacteriophage in the genus Neisseria (9, 59, 68, 69, 70, 78). With the exception of the report by Stone et al. (78), Phelps

(60) attributed all of the other reports to enzyme or bacteriocin activity rather than phage.

Stone et al. (78) isolated phage (designated neisseriaphage A) from a chromogenic strain of Neisseria which they identified as N. perflava. Electron micrographs reportedly revealed phage particles with an oval head of approximately 50 x 70 nm and a straight tail of approximately 20 x 160 nm, but electron micrographs did not accompany the report. Thirty-five strains of various species of Neisseria were tested for susceptibility to neisseriaphage A, and none was found to be susceptible.

Phelps (60) isolated and described 18 bacteriophage for the chromogenic species N. perflava, N. flavescens and N. flava. Electron micrographs of selected phage reportedly revealed phage particles with a polyhedral head approximately 700 Å in diameter, a tail which was approximately 1500 Å long and 150 Å wide, with a retractable tail sheath. Phage from another group were the same shape but about 10% larger. Electron micrographs did not accompany the report. Each phage was reported to have a very limited host range.

Cary and Hunter (10) later reported the isolation of five distinct bacteriophage from N. meningitidis strains. Electron micrographs reportedly revealed head-like particles similar to those described by Stone et al. (78), but no tail-like structures were observed. Electron micrographs

did not accompany the report. Some of the phage were active against other strains of N. meningitidis, but none was active against other Neisseria species or Mima polymorpha.

Salmonella pullorum as a Control Organism

Salmonella pullorum, a member of the Family Enterobacteriaceae, is a non-motile, gram negative rod measuring 0.3 to 0.6 by 0.8 to 2.5 μm (7). S. pullorum is very closely related to S. gallinarum, the causative agent of fowl typhoid (3). S. pullorum was discovered in 1899, and in 1900 was found to be the causative agent of pullorum disease, or bacillary white diarrhea (B.W.D.) of chicks (3, 93).

S. pullorum was selected as a control organism for three major reasons:

1. Both N. gonorrhoeae and S. pullorum have a %GC near 50. Although the exact %GC for S. pullorum is unknown, Belozersky and Spirin (5) reported a %GC of 50.2 for S. gallinarum compared to 49.6 for N. gonorrhoeae.

2. S. pullorum had been reported to contain cryptic plasmids (55). The organism therefore served as a positive plasmid-containing control.

3. S. pullorum had been investigated extensively in this laboratory, and many of the techniques utilized for N. gonorrhoeae research were modifications of the techniques previously utilized for S. pullorum research (54, 55, 77).

MATERIALS AND METHODS

Bacteria

Strains of N. gonorrhoeae mentioned in this thesis were obtained from two sources. Strains P-1 and 2686 were obtained from the Neisseria Research Unit, Venereal Disease Research Laboratory, Center for Disease Control, Atlanta, Georgia 30333. Strains 24 and 27 through 38 were clinical isolates obtained from the Bureau of Laboratories, Michigan Department of Public Health, Lansing, Michigan 48914.

S. pullorum strains MS53 and MS83 were obtained from the stock collection of Dr. D. E. Schoenhard.

Identification of N. gonorrhoeae

Gram negative, oxidase and catalase positive diplococci that produced acid from glucose and failed to produce acid from lactose, maltose and sucrose were identified as N. gonorrhoeae. These criteria enabled differentiation of N. gonorrhoeae from other Neisseria species, Herellea vaginicola and Mima polymorpha (46, 81, 82).

Taxo N Discs (BBL) and N, N-Dimethyl-p-phenylenediamine monohydrochloride were utilized to test for oxidase

production. Non-reagent grade hydrogen peroxide solution was used to test for catalase production.

Media

Distilled water which had subsequently passed through a Bantam Demineralizer (Barnstead Still & Sterilizer Co.) was utilized for the preparation of all media and reagents.

Gonococci were routinely cultivated on "chocolate" agar of the following formula:

	grams/liter
Bacto Brain Heart Infusion (Difco)	37.0
Special Agar - Noble (Difco)	17.5
Bacto Hemoglobin (Difco)	10.0
Bacto Yeast Extract (Difco)	3.0

"Chocolate" agar plates (BHCA plates) were stored at 4 C in sealed plastic bags. Plates were brought to 36 C prior to inoculation with N. gonorrhoeae.

The medium used to study colonial morphology was the same type used by Swanson et al. (80); it differed from the type used by Kellogg et al. (32, 33) and Jephcott and Reyn (24). The medium consisted of GC Agar Base (BBL) supplemented with IsoVitaleX Enrichment (BBL). Approximate final concentrations of ingredients were as follows:

	grams/liter
Polypeptone	15.0
Agar	10.0
Sodium chloride	5.0
Dipotassium phosphate	4.0
Corn starch	1.0
Monopotassium phosphate	1.0
Glucose	1.0
Cysteine hydrochloride	0.259
L-glutamine	0.1
L-cystine	0.011
Adenine	0.01
DPN oxidized (Coenzyme I)	0.0025
Cocarboxylase (thiamine pyrophosphate)	0.001
Guanine hydrochloride	0.0003
Ferric nitrate	0.0002
p-Aminobenzoic acid	0.00013
Vitamin B_{12}	0.0001
Thiamine hydrochloride	0.00003

It should be noted that the final concentration of agar was only 1%, necessitating the use of extreme care when inoculating these plates. Following solidification the plates (hereafter referred to as GCISO plates) were incubated for one hour at 36 C to reduce the moisture content and stored at room temperature in sealed plastic bags.

Screw-cap tubes of cystine trypticase agar (CTA Medium; BBL) containing 1% carbohydrate were utilized for determinations of carbohydrate metabolism (81, 82). Stock carbohydrate solutions were autoclaved separately and added aseptically to the sterile CTA Medium prior to solidification of the agar. Tubes were then tightly capped and stored at room temperature.

A modification of the biphasic flask system of Gerhardt and Hedén (20) was used to obtain broth cultures. The system consisted of a 250-ml Erlenmeyer flask containing 50 ml of solid medium overlaid with 25 ml of a clear liquid medium. Solid and liquid media were prepared according to the following formula:

	grams/liter
Solid medium	
Bacto Starch Agar (Difco)	25.0
Bacto Dextrose (Difco)	2.0
Liquid Medium	
Bacto Heart Infusion Broth	25.0
Dibasic sodium phosphate, anhydrous	11.9
Bacto Yeast Extract (Difco)	3.0
Monobasic potassium phosphate, anhydrous	2.2

A minimum of one hour prior to inoculation of the biphasic flask system (BFS), the liquid medium was aseptically added to a foam-plugged flask containing the solid

medium. The BFS was then equilibrated in a Gyrotory Water Bath Shaker (New Brunswick Scientific) set at 36 C and approximately 150 rpm.

The inoculum was prepared by suspending two or three loops of cells from a 24-hour plate culture in 1 ml of the liquid medium. A bacteriological loop approximately 3 mm in diameter was used. No attempt was made to accurately standardize the amount of inoculum. Following inoculation, the system was sealed with Parafilm "M" (Marathon) and incubated in the same water bath shaker.

The use of a phosphate-buffered liquid medium eliminated any need to adjust the pH of the medium following addition of antibiotic solutions, curing agents or other reagents. The final pH of the equilibrated system was 7.4, the optimal pH for N. gonorrhoeae.

Estimates of cell concentrations were based upon the viable cell counts obtained by counting the colonies on agar plates 24 to 48 hours after they were spread with appropriate dilutions of BFS cultures. Sterile BFS liquid medium was utilized in the preparation of dilutions.

Chemicals

General chemicals were of reagent grade and were purchased from standard commercial sources. Special chemicals and their sources are listed in Table 1.

Table 1. Special chemicals and sources.

Chemical	Source
Adenine-8-^{14}C	The Radiochemical Centre Amersham/Searle Chicago, Illinois
Bovine Albumin, crystallized	Pentex Biochemicals Kankakee, Illinois
Cesium Chloride, optical grade	Schwarz/Mann Orangeburg, New York
Deoxyribonucleic acid, from calf thymus, Type 1, sodium salt, highly polymerized	Sigma Chemical Co. St. Louis, Missouri
Ethidium bromide (2,7-diamino-10-ethyl-9-phenylpenanthridium bromide), B grade	Calbiochem Los Angeles, California
Lysozyme, crystallized, egg white	Armour Pharmaceutical Co. Kankakee, Illinois
POPOP; 1,4-Di[2-(5-Phenyloxazolyl)]-benzene, scintillation grade	Calbiochem Los Angeles, California
PPO; 2,5-Diphenyloxazole, scintillation grade	Calbiochem San Diego, California
Ribonuclease 5x cryst., (bovine pancreas--salt free), A grade	Calbiochem Los Angeles, California
Triton X-100 (Octyl Phenoxy Polyethoxyethanol)	Sigma Chemical Co. St. Louis, Missouri

Buffers and Dialysis

TES buffer, pH 8.0, contained 0.05 M Tris (hydroxymethyl) aminomethane, 0.005 M disodium ethylenediaminetetraacetate (EDTA), and 0.05 M NaCl.

Phosphate buffer, pH 7.4, contained 0.084 M Na_2HPO_4 and 0.016 M KH_2PO_4.

Dialysis was performed with sterile 1/4" dialyzer tubing (Arthur H. Thomas Co.) which had been boiled in 0.5 M EDTA, pH 7.0, for 10 minutes and subsequently autoclaved in 0.05 M Tris, pH 8.0.

Maintenance of Agar Plate Cultures

Inoculated agar plates were incubated in a candle jar at 36 C. Wet paper towels were placed in the bottom of the candle jar to provide moisture in the atmosphere.

Cultures used for colonial morphology purposes were subcultured daily onto GCISO plates. Other cultures were subcultured every second day.

Temporary Preservation of Stock Cultures

The broth from an 11-hour BFS culture was aseptically transferred to a sterile 50-ml plastic centrifuge tube, and centrifuged for 20 min at 10,000 rpm and 4 C in a Sorvall Model RC-2 Automatic Refrigerated Centrifuge.

The cells were resuspended in 4 ml of a sterile broth of the following formula:

	grams/liter
Proteose Peptone No. 3 (Difco)	15.0
K_2HPO_4	4.0
KH_2PO_4	1.0
Cornstarch (non-reagent grade; Argo Brand)	1.0
NaCl	0.5

The cell suspension was then aseptically transferred to a screw-cap test tube containing 1 ml of sterile glycerol, thoroughly mixed utilizing a Super-Mixer (Matheson Scientific) and stored at -25 to -30 C. The cells were thus stored in a 20% (v/v) glycerol broth similar to that described by Sparling (74).

Recovery of stock cultures was accomplished by streaking a loop of the semi-frozen mixture across a prewarmed (36 C) agar plate and incubating in a candle jar at 36 C.

This method of preservation is not recommended for periods in excess of three months, as the recovery rate dropped severely beyond that time interval. A more efficient method for preservation of N. gonorrhoeae has been described (87), but was not utilized during this investigation.

Determination of Colony Type

Twenty-four hour cultures on GCISO plates were used to determine colony types. The plates were examined with a Cycloptic Stereoscopic Microscope (American Optical). The colonies were illuminated from the underside with a Cyclospot Illuminator (American Optical) and the matte-ground opal mirror of the Transilluminator Base (American Optical). The colonies received additional illumination from the top by the available light within the room.

Utilizing the stereoscopic microscope and a sterile inoculating needle, specific colony types were transferred daily.

Electron microscopy

Negative staining was accomplished using 1% Phosphotungstic acid, pH 6.0, 2% ammonium molybdate, pH 6.8, or 2% aqueous uranyl acetate, pH 3.5. A single colony was removed from the surface of an agar plate and suspended in a small drop of sterile demineralized water. A Pasteur pipette was then used to transfer the cell suspension to a collodion- or formvar-coated electron microscope grid. After 30 seconds the excess fluid was removed with a piece of Whatman No. 1 filter paper. A drop of the stain was then placed on the grid and, after a specific time interval, was removed with another piece of filter paper.

The grids were examined with a Philips 300 and/or a Hitachi HU-11 electron microscope.

Antimicrobial Agent Sensitivity Testing

Sensitivity of gonococcus strains to antibiotics and Hg^{++} was determined by one or both of the following criteria:

1. The ability of the strain to multiply in a normal manner on solid medium containing known concentrations of antimicrobial agents.

2. Zones of growth inhibition surrounding paper discs impregnated with antimicrobial agents. Bacto Sensitivity Disks for Antibiotics (Difco) and sterile Whatman No. 1 filter paper discs impregnated with specific quantities of antibiotics were utilized. No attempt was made to standardize inoculum size, and zones of growth inhibition were used only as qualitative guides to relative sensitivity or resistance.

Stock solutions of antibiotics were prepared with phosphate buffer, pH 7.4, were passed through filters (Millipore Filter Corp.) having a 0.45 μm pore size, and were stored in 1 ml aliquots at -25 to -30 C. Thawed stock antibiotic solutions were stored at 4 C for a maximum of 7 days.

When antibiotics were incorporated into media, aliquots of the stock antibiotic solutions were added following autoclaving and subsequent cooling of the media to approximately 50 C.

Curing Procedure

Ethidium bromide, acridine orange and sodium dodecyl sulfate (SDS) were utilized in attempts to cure gonococcus strains of their multiple antibiotic resistance.

Equal concentrations of the curing agent were incorporated into the solid and liquid media of a BFS. Aliquots of the stock curing agent solutions were added following autoclaving and subsequent cooling of the media to approximately 50 C. Stock solutions of curing agents were prepared with phosphate buffer, pH 7.4, and were subsequently passed through filters having a 0.45 μm pore size. Stock solutions of curing agents were stored at 4 C for a maximum of 7 days.

For each experiment a curing agent-containing BFS and a control BFS were inoculated equally and were incubated in a water bath shaker set at 36 C and approximately 150 rpm. BFS's containing ethidium bromide were incubated in the dark to prevent any adverse effect that light may have upon the biological activity of the dye.

After 11 hours incubation serial dilutions of the BFS cultures were prepared and BHCA plates were spread. Plain BHCA and antibiotic-containing BHCA plates were used;

the antibiotic sensitivity profile of each gonococcus strain determined the types and concentrations of antibiotics incorporated into the BHCA plates.

Colony counts were made after 24 to 48 hours incubation. The colony count data were analyzed for indications of spontaneous curing in the control culture and a combination of spontaneous and actual curing in the test culture.

Radioactive Labeling

Four hours after inoculation, 10 ml of the broth from a BFS were aseptically transferred to a 250-ml Erlenmeyer flask containing 25 ml of prewarmed (36 C) and well-mixed "labeling broth." The inoculated "labeling broth" was incubated for an additional 7 hours in a water bath shaker set at 36 C and approximately 150 rpm.

"Labeling broth" was prepared from BFS liquid medium. Inoculated "labeling broth" contained a final concentration of 0.228 µg of ^{14}C- adenine/ml (0.1 µCi/ml) and 1.14 µg of unlabeled adenine/ml, approximating the maximum concentrations used by Kingsbury and Duncan(38) to label the nucleic acids of N. meningitidis. The "labeling broth" utilized for S. pullorum MS83 also contained a final concentration of 7.0 µg of uridine/ml because this strain was auxotrophic for uridine.

Stock solutions of ^{14}C-adenine, unlabeled adenine and uridine were prepared with phosphate buffer, pH 7.4, and stored over chloroform at 4 C.

Estimates of uptake efficiency were based upon scintillation counts obtained from 0.1 ml aliquots of the 11-hour "labeling broth" cultures and 0.1 ml aliquots of the supernatant fluid following 20 min centrifugation of the 11-hour "labeling broth" cultures at 10,000 rpm and 4 C in a Model RC-2 Sorvall.

Scintillation Counting

Samples for scintillation counting were collected or spotted on 3/4" squares of Whatman No. 1 filter paper. Dried filter paper squares were placed in plastic scintillation vials containing 5 ml of scintillation fluid, and were counted in a Packard Model 2002 Tri-Carb Liquid Scintillation Spectrometer.

The scintillation fluid was prepared by adding 5 gm of PPO and 100 mg of POPOP to one gallon of toluene.

Preparation of Bacterial Lysates

The entire 35 ml of an 11-hour "labeling broth" culture was transferred to a sterile 50-ml plastic centrifuge tube and centrifuged for 20 min at 10,000 rpm and 4 C in a Model RC-2 Sorvall.

Cell lysates were prepared by a modification of the techniques of Clewell and Helinski (14). The cell pellet was resuspended in 1 ml of cold 25% sucrose in 0.05 M Tris,

pH 8.0, by gentle vortexing. The resuspended cells were transferred by Pasteur pipette to a sterile 15-ml Pyrex centrifuge tube, quick-frozen in a dry ice and ethanol bath, and stored at -25 to -30 C until required.

The tube contents were allowed to thaw at room temperature, after which 0.2 ml of cold lysozyme solution (5 mg/ml of 0.25 M Tris, pH 8.0) were immediately added. Lysozyme solution was prepared on the day of use. The tube was then placed in an ice bath for 5 minutes. Four-tenths ml of cold 0.25 M EDTA, pH 8.0, were then added, and the tube was placed in an ice bath for 5 minutes. Next 1.6 ml of cold lysing mixture were added, and the tube was placed in an ice bath for 15 minutes. The lysing mixture contained 1% (v/v) Triton X-100, 1% (w/v) sodium deoxycholate, 0.625 M EDTA in 0.05 M Tris, pH 8.0.

The suspension was then drawn into and gently expelled from a 1-ml pipette a total of 10 times to insure mixing of the ingredients and to shear the chromosomal DNA. The tube was then returned to the ice bath for 10 additional minutes.

When necessary, an RNAse step was included at this point in the procedure. Two-tenths ml of pretreated RNAse solution (0.68 mg/ml of TES buffer) were added to the tube. The tube was then incubated for 30 min in a 36 C waterbath. The final RNAse concentration was 40 µg/ml. The RNAse

solution was pretreated at 75 to 85 C for 15 minutes to inactivate any contaminating DNAse that might have been present.

The crude lysate was then centrifuged for 30 mintues at 20,000 rpm and 4 C in a Model RC-2B Sorvall. This step pelleted approximately 95% of the chromosomal DNA (14).

This procedure yielded relatively clear and viscous lysates of N. gonorrhoeae, S. pullorum and E. coli.

Alkaline Sucrose Gradients

Alkaline sucrose gradients were prepared by a modification of the procedure described by Olsen and Schoenhard (55). A 0.5 ml aliquot of bacterial lysate was layered onto a 5.2-ml 20 to 31% (w/v) linear alkaline sucrose gradient prepared with 1.0 M NaCl, 0.3 M NaOH and 0.01 M EDTA, pH 12.0. Gradients were prepared in 1/2" x 2" cellulose nitrate tubes (Beckman) which had been presoaked for a minimum of one hour in 100 µg denatured calf thymus DNA and 1.0 mg bovine albumin/ml of TES buffer. Denaturation was accomplished by placing a tube of DNA-TES solution into a boiling water bath for 10 minutes, after which the tube was placed immediately into an ice bath to prevent renaturation.

Gradients were placed in a SW50L Spinco rotor and centrifuged for 90 minutes at 50,000 rpm and 15 C in a Beckman Model L or L3-50 Ultracentrifuge.

A Beckman Recovery System was used to collect 5-drop fractions from the bottom of the cellulose nitrate tubes. The fractions were collected directly onto prenumbered 3/4" filter paper squares. The filter paper squares were dried in an oven set at 65 to 70 C, after which they were washed twice in cold 5% (w/v) Trichloroacetic acid (TCA), once in cold 95% (v/v) ethanol, and once in cold anhydrous ether. Two-hundred to 250 ml of reagent were used for each washing. The filter paper squares were thoroughly dried prior to counting.

Dye-buoyant Density Gradients

Cesium chloride-ethidium bromide gradients were prepared by a modification of the procedure described by Olsen and Schoenhard (55). Sufficient TES buffer was added to the lysate to bring the total volume to 5.7 ml. This mixture was added to a vial containing 0.5 ml of ethidium bromide solution (5 mg/ml of TES buffer) and 6.0 grams of finely crushed, anhydrous cesium chloride. The mixture was gently swirled until all of the cesium chloride was dissolved and then transferred to a 5/8" x 2 1/2" polyallomer tube (Beckman). Polyallomer tubes were pretreated by boiling for 15 min in TES buffer and subsequent soaking in 100 µg bovine albumin/ml of TES buffer for a minimum of two hours.

The mixture was overlaid with light mineral oil to completely fill the tubes, and the tubes were tightly capped. Equilibrium centrifugation was accomplished at 44,000 rpm and 15 C for 30 hours in a Type 50 Spinco rotor and a Beckman Model L or L3-50 Ultracentrifuge.

Seventy 10-drop fractions were collected directly onto prenumbered 3/4" filter paper squares by puncturing the bottom of the polyallomer tube with a locally-manufactured fraction recovery system employing a 24-gauge needle.

Treatment of filter paper squares was as described for alkaline sucrose gradients.

When pooled fractions of plasmid DNA were required the fractions were collected in a sterile glass vial rather than on filter paper squares. These pooled fractions were then transferred by Pasteur pipette to a sterile piece of pretreated dialysis tubing and dialyzed overnight at 4 C in the dark against three 1000-ml changes of TES buffer to remove the cesium chloride and ethidium bromide.

RESULTS

Biphasic Flask System Cultures

BFS cultures of N. gonorrhoeae in Delong 250-ml side-arm flasks yielded typical growth curves when monitored hourly with a Spectronic 20 spectrophotometer (Bausch & Lomb). Figure 1 represents a growth curve obtained from a BFS culture in a Delong side-arm flask. N. gonorrhoeae strain P-1 cells harvested from a 36-hour BHCA plate were used as inoculum. As previously mentioned, no attempt was made to accurately standardize the inoculum size. Based upon viable cell counts, the inoculum was estimated to be in the order of 10^9 cells.

The duration of the lag phase was influenced by such factors as the number and age of the cells utilized as inoculum, and the type of solid medium from which they were transferred. The variable lag period was followed by a period of exponential growth, during which a minimum of three to four doublings occurred. Maximum cell concentrations occurred 10 to 12 hours after inoculation, and viable cell counts as high as 7×10^8 organisms/ml were achieved.

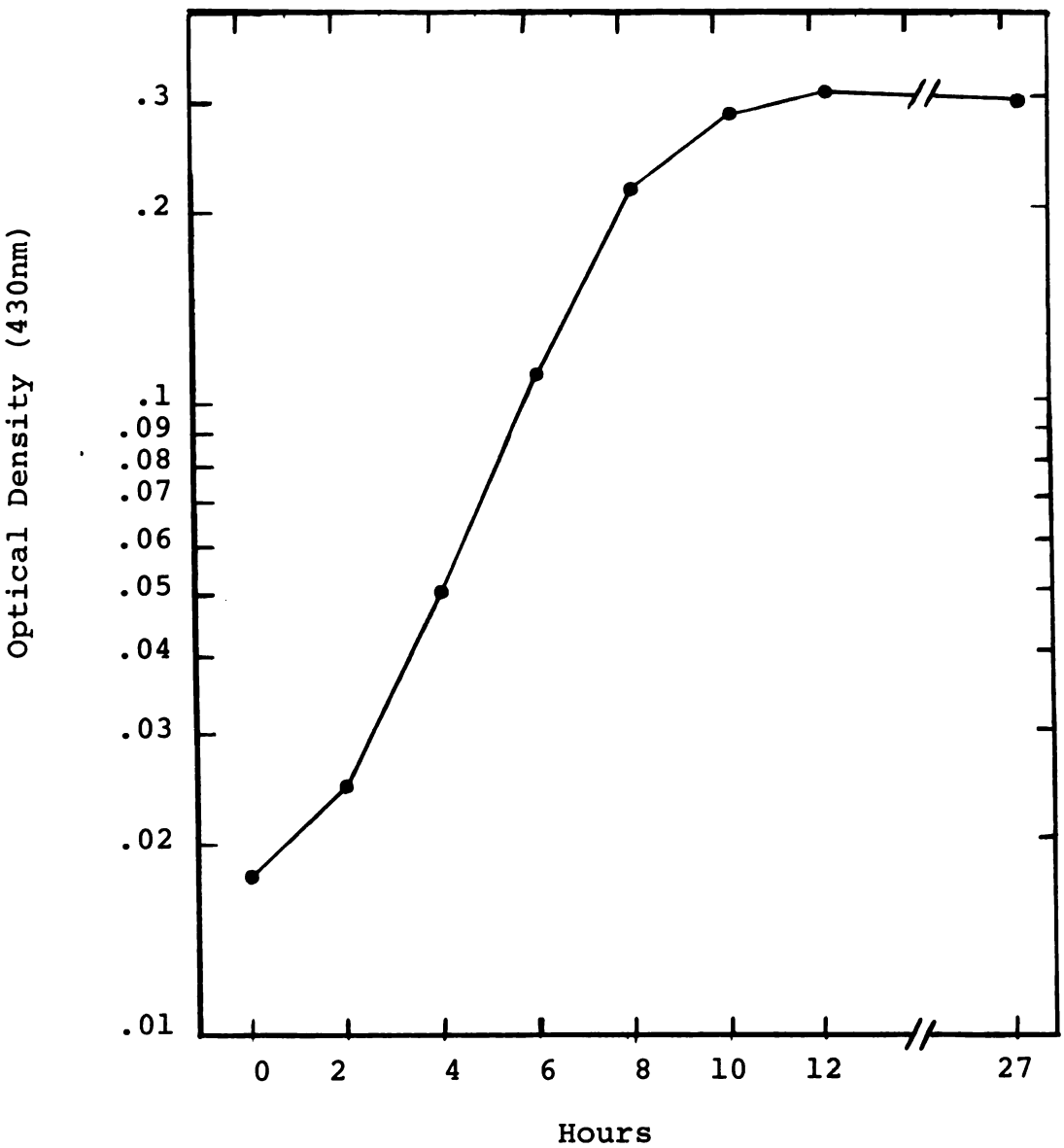

Figure 1. A growth curve of N. gonorrhoeae. A biphasic flask system in a 250-ml Delong side-arm flask was inoculated with N. gonorrhoeae strain P-1 cells harvested from a 36-hour BHCA plate.

Accurate viable cell counts were difficult to achieve due to the tendency of N. gonorrhoeae to clump when cultivated in liquid medium. Serial dilutions of BFS cultures were thoroughly vortexed, but some of the colonies undoubtedly arose from more than a single cell. Consequently, the 7×10^8 figure most likely represents an underestimate of maximum cell concentration.

Figure 2 represents the correlation between optical density and viable cell count. Data for Figure 2 were obtained from two BFS cultures of N. gonorrhoeae strain P-1.

Variations between optical density readings and viable cell counts were thought to be due to clumping of cells and optical differences between the Delong side-arm flasks.

Colonial Morphology

In general, colony types 1, 2 and 3 appeared as previously described in the Literature Review. Type 4 colonies were not observed.

Type 1 and type 2 colonies were approximately one-half the diameter of the type 3 colonies. Type 1 and type 2 colonies appeared darker in color and less granular than type 3 colonies. Type 2 colonies could readily be distinguished from type 1 colonies by a pronounced dark ring encircling the colonies. When touched with an inoculating

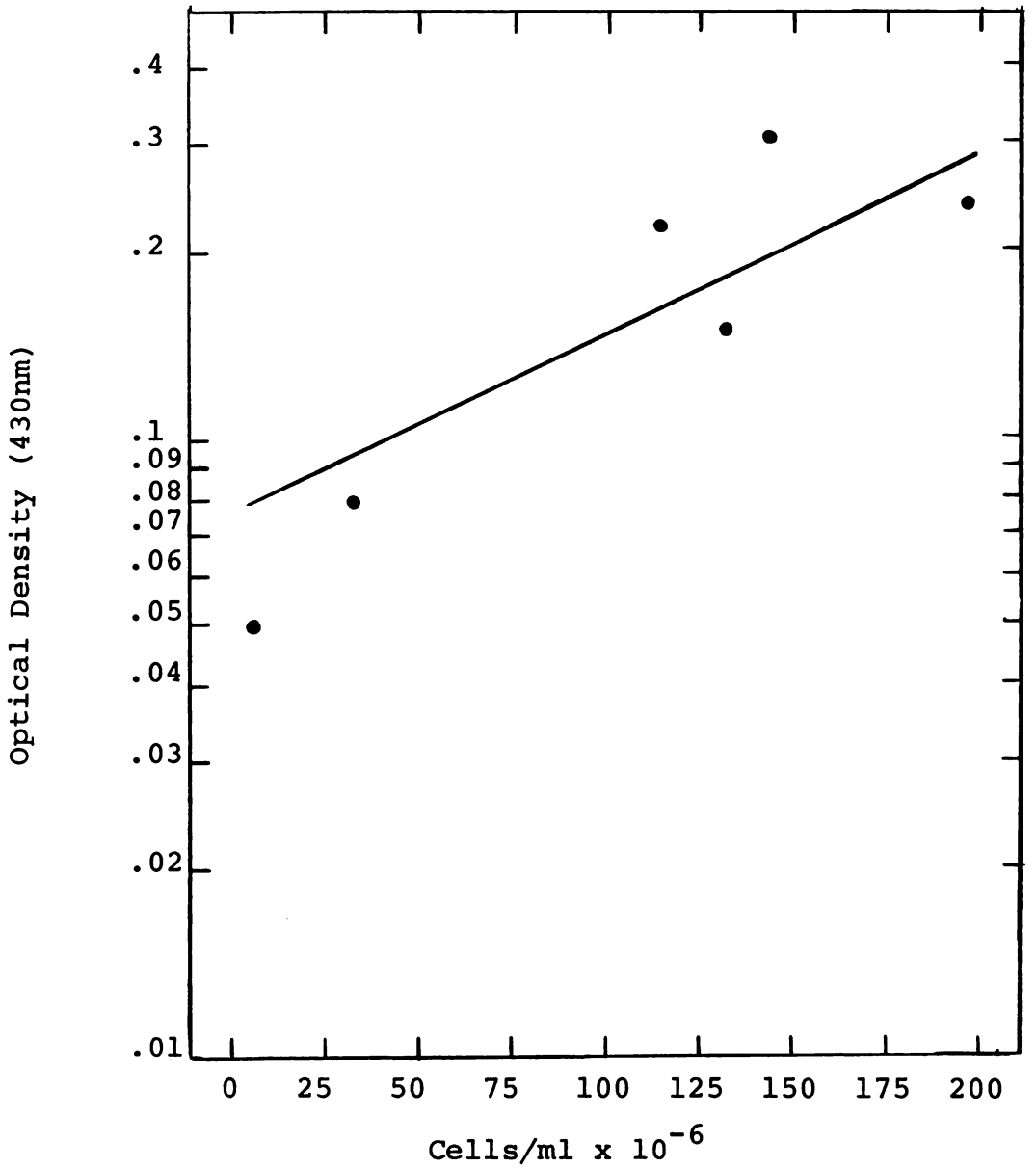

Figure 2. Correlation between optical density readings and viable cell counts. The regression line was calculated by the method of least squares.

needle type 3 colonies were watery, whereas colony types 1 and 2 were friable. Type 2 colonies were more friable than type 1.

No attempt was made to study or quantitate the rates at which one colony type converted to another. Some general observations will be mentioned, however.

Type 3 colonies were extremely stable; no other colony type appeared upon subculture of a type 3 colony.

Type 2 colonies were relatively stable; only rarely did a type 3 colony appear upon subculture.

Type 1 colonies were relatively unstable; many type 3 colonies arose upon subculture. This observation suggested that the markers determining colony type may be located on a plasmid of some sort, and the conversion of colony type 1 to colony type 3 may represent spontaneous loss of the plasmid. This possibility was investigated by means of density gradient centrifugation.

Attempts to obtain BFS cultures of pure type 1 colony-producing cells were unsuccessful. When GCISO plates were spread with samples of 11-hour BFS cultures inoculated with type 1 colonies, approximately 1/3 of the resulting colonies were type 1 and 2/3 were type 3.

Only type 3 colonies arose from GCISO plates spread with samples of 11-hour BFS cultures inoculated with type 3 colonies.

Figure 3 shows the appearance of colony types 1 and 3 when observed with a stereoscopic microscope and illuminated in the manner described in Materials and Methods.

Electron Microscopy

Extremely short intervals of time were required to prevent overstaining of the gonococcal cells. The best results were obtained with uranyl acetate when the stain was removed immediately after addition to the grid. Time intervals of 5 seconds or less produced the most favorable results with PTA and ammonium molybdate. Some variation of staining was noted among cells on the same grid regardless of the type of negative stain utilized.

Negative staining revealed the typical diplococcus morphology, an extremely convoluted cell surface, and gonococcal pili when such appendages were present. Ideal staining of the pili was usually accompanied by densely stained cells; therefore, varying degrees of "dodging" were necessary during the printing process.

Pili were observed on some, but not all, of the cells from type 1 colonies. Pilus length was obviously variable, but the diameter seemed constant. No attempt was made to measure either length or width of the pili. Tangled pili were frequently observed, and pili from the same or neighboring cells were frequently intertwined forming a braid-like structure. Uranyl acetate-stained pili appeared more convoluted than PTA-stained pili.

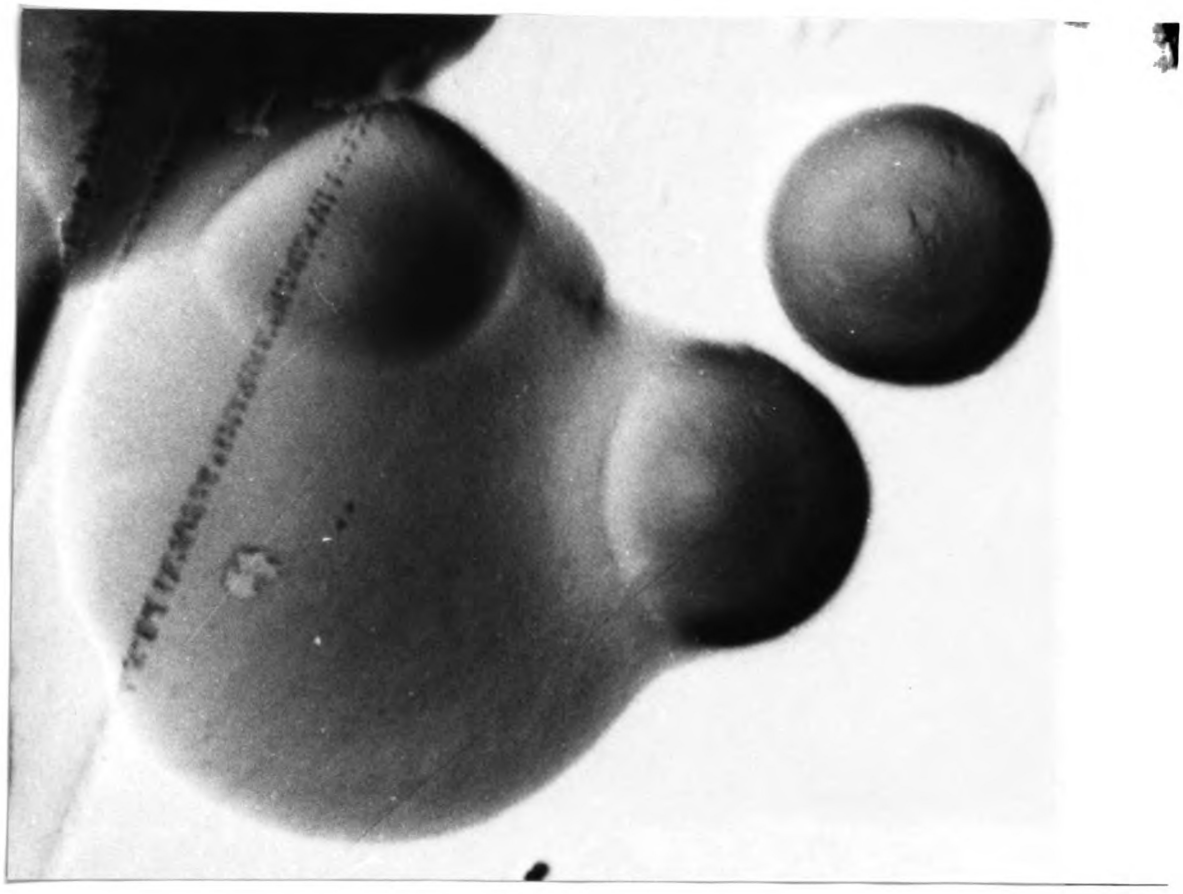

Figure 3. Gonococcus colony types 1 and 3 on GCISO medium. N. gonorrhoeae strain 2686 colony types 1 (the small, dark colonies) and 3 (the large, light colony) are shown. The type 3 colony probably represents an outgrowth of one or both of the type 1 colonies that it is in contact with. The colonies were illuminated in the manner described in Materials and Methods and photographed through a stereoscopic microscope. The approximate magnification is 76x.

All cells from type 2 colonies appeared to be piliated. No differences were observed between type 1 and type 2 pili.

Pili were not observed on cells from type 3 colonies.

Figures 4, 5 and 6 are representative electron micrographs which show the typical appearance of negatively-stained gonococcal cells and pili.

Resistance to Antimicrobial Agents

Most of the freshly-isolated strains of N. gonorrhoeae tested were found to be resistant to low levels of at least one antibiotic. Sensitivity profiles of 13 representative strains are presented in Table 2.

All 13 strains were resistant to 0.1 µg of tetracycline (TET)/ml. Most strains tested for sensitivity to 0.1 u of penicillin (PEN)/ml, 5 µg of kanamycin (KAN)/ml, or 5 µg of neomycin (NEO)/ml were found to be resistant.

All strains tested for sensitivity to 2 µg of erythromycin (ERY)/ml and 5 µg of chloramphenicol (CHL)/ml were found to be susceptible.

Three of the strains were found to be resistant to 100 µg of streptomycin (STR)/ml.

Strains 24 and 37 were subsequently examined for physical evidence of plasmids because each of these strains was resistant to at least five antibiotics.

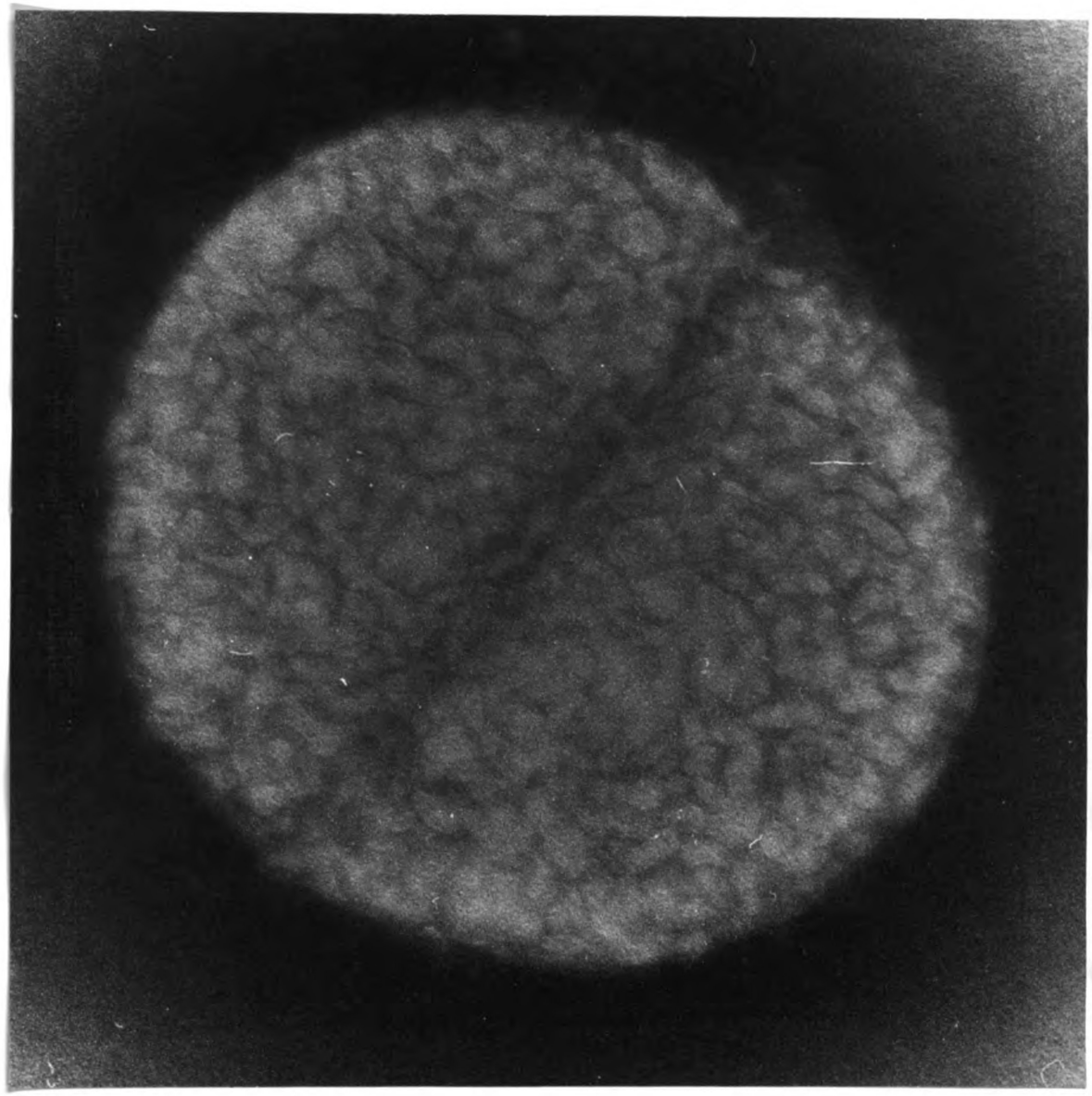

Figure 4. A negatively-stained gonococcus cell. A N. gonorrhoeae strain 28 cell in the process of division. The cell was negatively-stained by 5 seconds exposure to 1% phosphotungstic acid, pH 6.0, and was photographed through a Hitachi HU-11 electron microscope. The approximate magnification is 142,000x.

Figure 5. Negatively-stained piliated gonococcus cells. These piliated cells from a N. gonorrhoeae strain 2686 type 1 colony were negatively-stained with 2% aqueous uranyl acetate, which had been immediately removed following its addition to the grid. No attempt was made to "dodge" the densely-stained cells during the printing process. The electron micrograph was taken with a Philips 300 electron microscope. The approximate magnification is 130,000x.

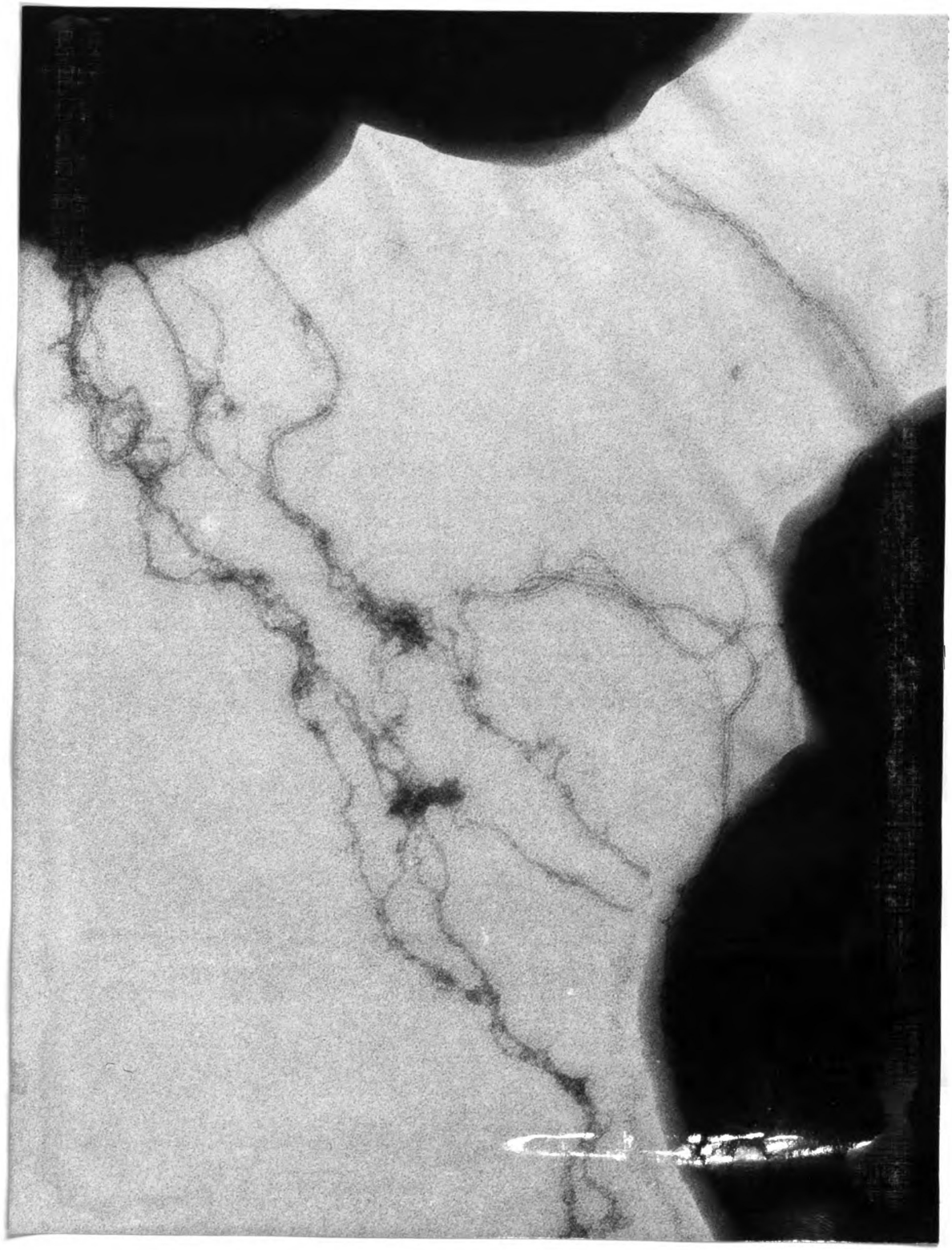

Figure 5.

Figure 6. Negatively-stained gonococcal pili. This piliated cell from a N. gonorrhoeae strain 2686 type 1 colony was negatively stained with 2% aqueous uranyl acetate, which had been immediately removed following its addition to the grid. The cell area was "dodged" during the printing process in an attempt to reveal the pili attachment sites. Terminal structures are apparent on several of the pili. The electron micrograph was taken with a Philips 300 electron microscope. The approximate magnification is 130,000x.

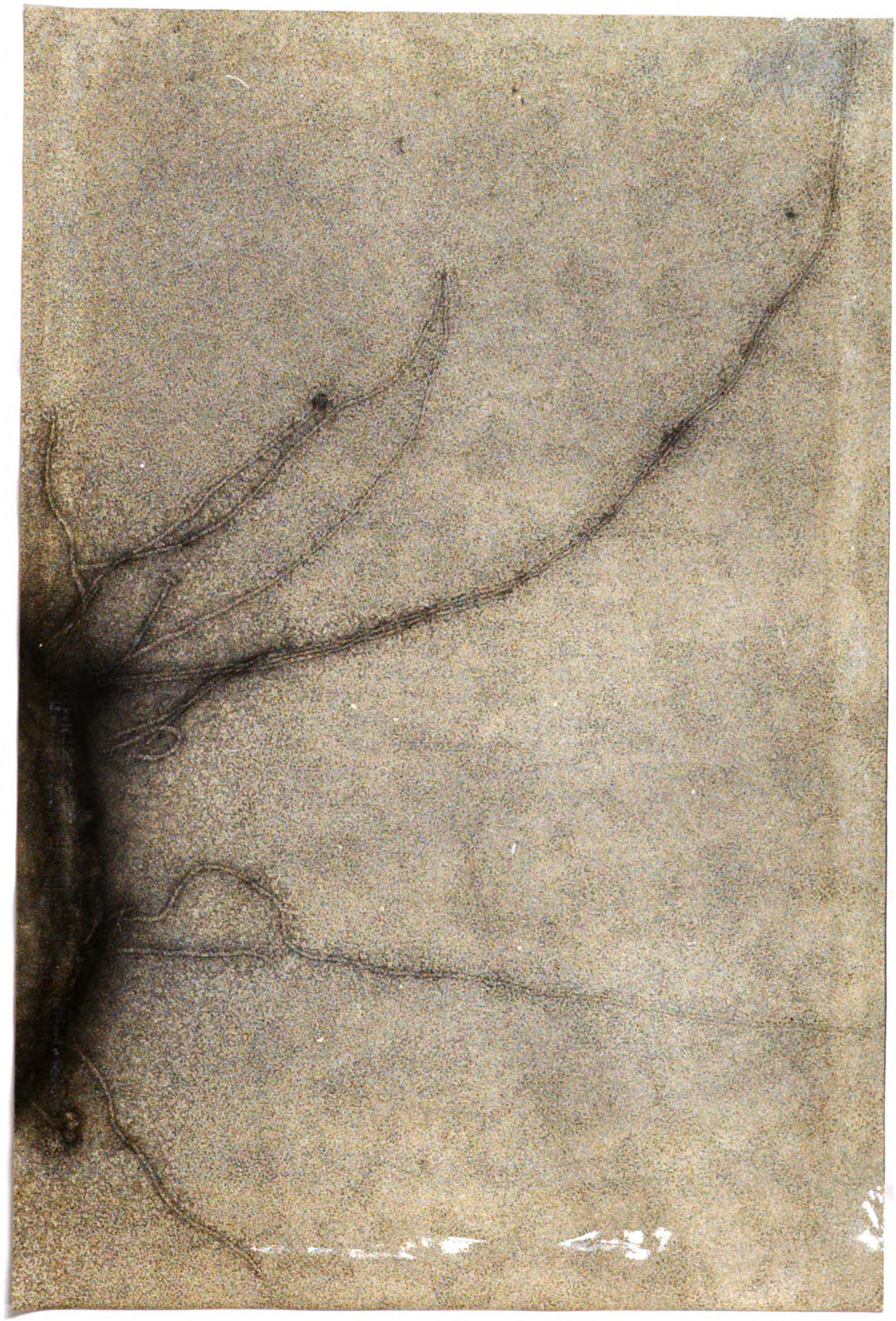

Figure 6.

Table 2. Antibiotic sensitivity profiles.

Strain	Quantity of antibiotic/ml																
	0.1 u PEN	1.0 u PEN	2.0 u PEN	0.1 µg TET	1.0 µg TET	4.0 µg TET	5.0 µg TET	2.0 µg STR	10 µg STR	25 µg STR	100 µg STR	5.0 µg KAN	30 µg KAN	5.0 µg NEO	30 µg NEO	5 µg CHL	2 µg ERY
24	R[a]	S[b]		R	R		S		R	R	R	R	S	R	S	S	S
27	R	S		R	R				R	R							
28	R	S		R	S	S		R	S			R		R		S	S
29	R	S		R	R	R	S	R	R	R		R	S	R	S	S	S
30	R	S		R	R	S		R	R			S		R		S	S
31	S			R	S			S				S		S		S	S
32	S			R	S				S								
33	R	S		R	R				S								
34	R	R	S	R	R				S								
35	S			R	S				R	S							
36	S			R	S				R	S							
37	R	R	S	R	R		S		R	R	R	R	S	R	S	S	S
38	R	S		R	R				R	R	R						

[a]Resistant

[b]Sensitive

Four strains of gonococci with various antibiotic sensitivity profiles (including strain 24) were tested for sensitivity to Hg^{++} ions using BHCA plates either containing mercuric chloride and/or overlaid with mercuric chloride-impregnated discs. All strains were resistant to 2×10^{-10} moles, but susceptible to 1×10^{-9} moles. The strains appeared to be equally susceptible, and there appeared to be no correlation between antibiotic sensitivity profiles and Hg^{++} sensitivity.

Curing Experiments

Numerous attempts were made to cure N. gonorrhoeae strains of their multiple antibiotic resistance. The results of these experiments were not reproducible and for this reason are not presented.

The experiments did furnish some information regarding the inhibitory effect of various curing agents on the growth of N. gonorrhoeae. Some variations in sensitivity were noted between strains.

Gonococcus strains utilized in these experiments were unaffected by 10^{-7} M ethidium bromide, but 4×10^{-6} M ethidium bromide resulted in greater than 99% inhibition of growth. An acridine orange concentration of 20 µg/ml also resulted in greater than 99% inhibition of growth. A 0.002% (w/v) concentration of sodium dodecyl sulfate caused 62% inhibition of growth.

Radioactive Labeling

Attempts to label N. gonorrhoeae DNA with ^{3}H-thymidine resulted in uptake efficiencies of 11.5% or less. These uptake efficiencies were considered to be inadequate for detection of small quantities of plasmid DNA.

^{14}C-adenine was subsequently used as a nucleic acid label. An average uptake efficiency of approximately 29% was achieved for N. gonorrhoeae strains and approximately 22% for S. pullorum.

In general, greater uptake efficiency was obtained when BFS's were inoculated with cells from a GCISO plate. These plates contained an adenine concentration of approximately 10^{-2} mg/ml. Cells transferred from such a plate were then starved of adenine in the BFS. When subsequently transferred into "labeling broth", which had an adenine concentration of 1.37×10^{-3} mg/ml, they took up the label well.

Alkaline Sucrose Gradients: Bacterial Lysates

Figure 7A depicts the fractions collected from an alkaline sucrose gradient of a S. pullorum MS83 lysate. The lysate had not been treated with RNAse. The uptake efficiency of the culture was approximately 35.5%. Approximately 8.3% of the estimated total counts per minute (cpm) added to the gradient was recovered. Three distinct peaks are evident.

Figure 7B depicts the fractions collected from an alkaline sucrose gradient of a lysate of N. gonorrhoeae strain 2686 (colony types 1 and 3). The lysate had been treated with RNAse. The 11-hour "labeling broth" culture contained approximately 31.0% type 1 colony-producing cells and 69.0% type 3 colony-producing cells. The uptake efficiency of the culture was approximately 30.3%. Approximately 6.5% of the estimated total cpm added to the gradient was recovered. Three peaks are evident.

Figure 7C depicts the fractions collected from an alkaline sucrose gradient of a lysate of N. gonorrhoeae strain 2686 (colony type 3). The lysate had been treated with RNAse. The uptake efficiency of the culture was approximately 16.9%. Approximately 5.6% of the estimated total cpm added to the gradient was recovered. One large peak and two relatively small peaks are evident. The two small peaks are in the same relative positions as the small peaks of Figure 7B.

When a lysate of N. gonorrhoeae strain 37 was centrifuged in an alkaline sucrose gradient, no evidence of rapidly-sedimenting DNA was obtained. The data are not presented.

N. gonorrhoeae strain 24 yielded conflicting alkaline sucrose data. One experiment, during which no RNAse step was performed, yielded no evidence of rapidly-sedimenting DNA. Another experiment, which included an

Figure 7. Alkaline sucrose gradients: bacterial lysates. Five-tenths ml of a ^{14}C-adenine-labeled bacterial lysate were layered onto a 5.2-ml, 20 to 31% (w/v) linear alkaline sucrose gradient and centrifuged in a SW50L rotor for 90 minutes at 50,000 rpm and 15 C. Fractions were collected onto filter paper squares, washed in TCA, ethanol and ether, and counted for radioactivity. (A) S. pullorum MS83, (B) N. gonorrhoeae strain 2686 (colony types 1 and 3), (C) N. gonorrhoeae strain 2686 (colony type 3).

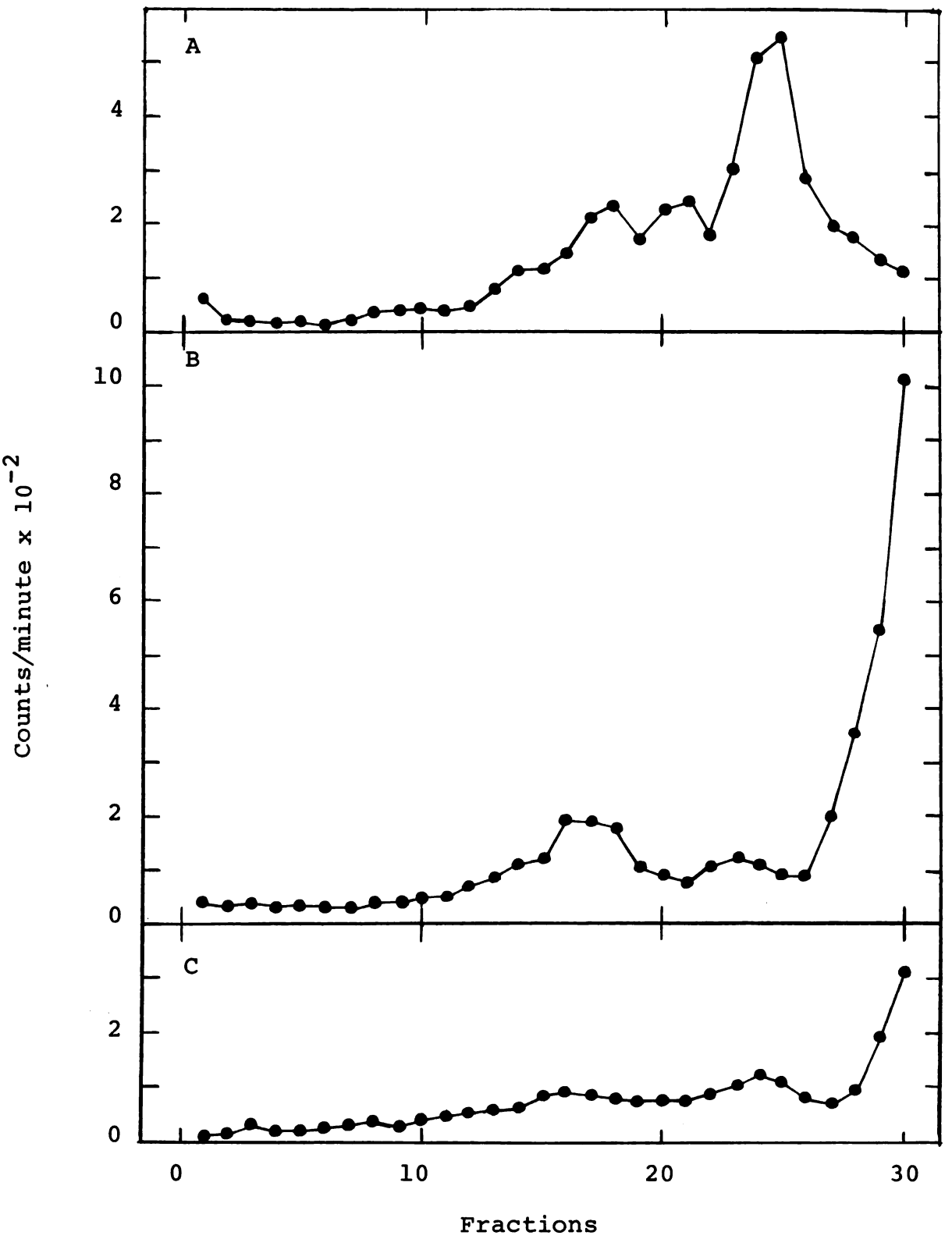

Figure 7.

RNAse step, yielded evidence of rapidly-sedimenting DNA six fractions below the larger peak. Time did not permit this conflict to be resolved, and the alkaline sucrose data for strain 24 are therefore not presented. It is possible that the two gradient experiments involved different colony types.

Dye-Buoyant Density Gradients

Figure 8 depicts the fractions collected from a cesium chloride-ethidium bromide gradient of a S. pullorum MS83 lysate. The lysate utilized in this particular experiment also yielded the alkaline sucrose gradient fractions depicted in Figure 7A. Two peaks are evident. The apex of the larger peak is located in fraction no. 50, and the apex of the smaller peak in fraction no. 36.

Similar data (not shown) were obtained for S. pullorum MS53. The apex of the larger peak was located in fraction no. 56, and the apex of the smaller peak in fraction no. 38.

Figure 9 depicts the fractions collected from a cesium chloride-ethidium bromide gradient of a lysate of N. gonorrhoeae strain 2686 (colony types 1 and 3). The lysate had not been treated with RNAse. The uptake efficiency of the culture was approximately 37.5%. Two distinct peaks are evident. The apex of the larger peak is

Figure 8. Cesium chloride-ethidium bromide gradient of S. pullorum MS83. A ^{14}C-adenine-labeled S. pullorum lysate was added to a cesium chloride-ethidium bromide mixture and centrifuged to equilibrium in a Type 50 rotor for 30 hours at 44,000 rpm and 15 C. Fractions were collected onto filter paper squares, washed in TCA, ethanol and ether, and counted for radioactivity.

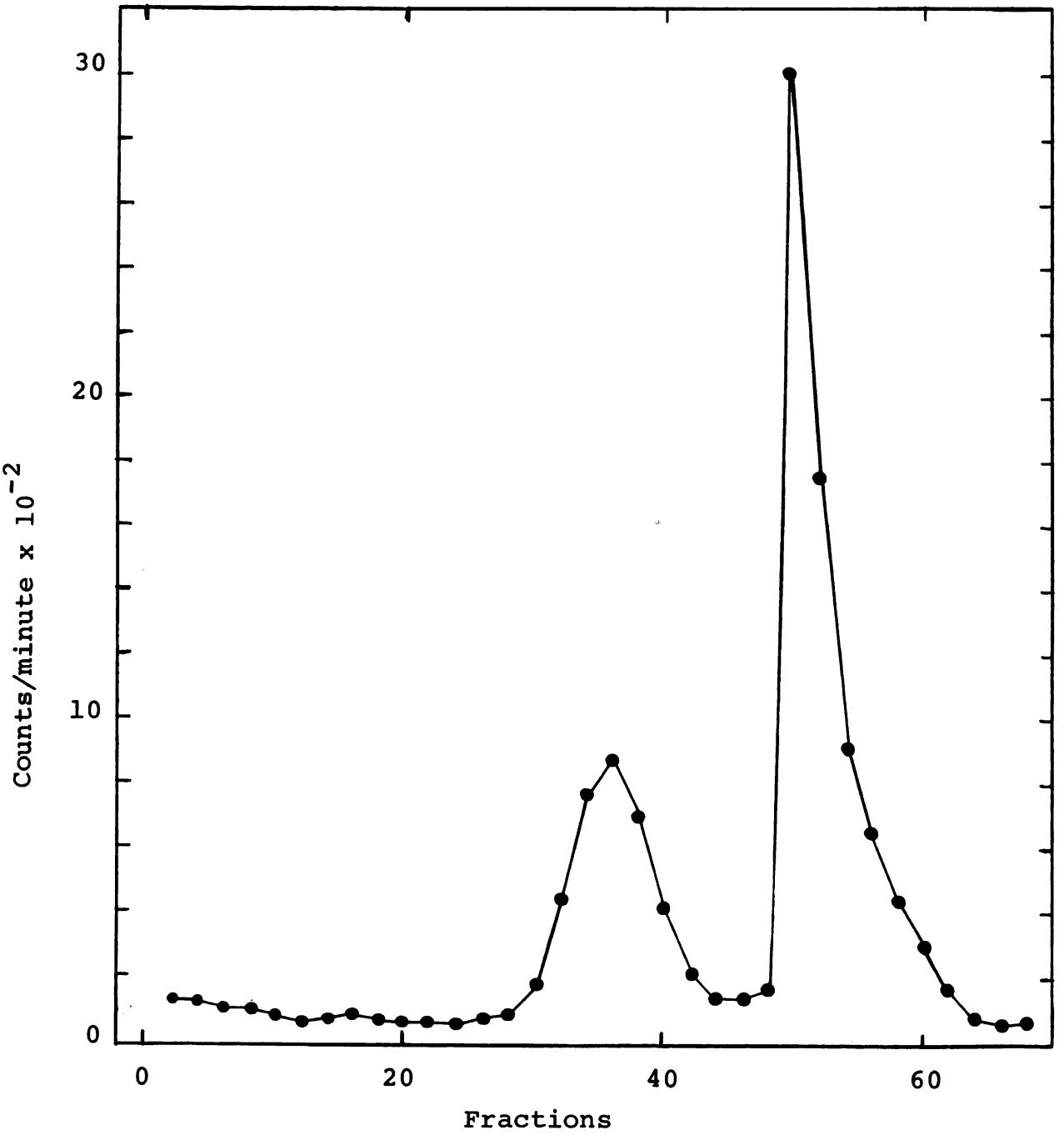

Figure 8.

Figure 9. Cesium chloride-ethidium bromide gradient of N. gonorrhoeae strain 2686 (colony types 1 and 3). A ^{14}C-adenine-labeled lysate of N. gonorrhoeae strain 2686 (colony types 1 and 3) was added to a cesium chloride-ethidium bromide mixture and centrifuged to equilibrium in a Type 50 rotor for 30 hours at 44,000 rpm and 15 C. Fractions were collected onto filter paper squares, washed in TCA, ethanol and ether, and counted for radioactivity. The data for fractions no. 2 through 14 are not shown; cpm decreased steadily from 8385 in fraction no. 2 to 1687 in fraction no. 14.

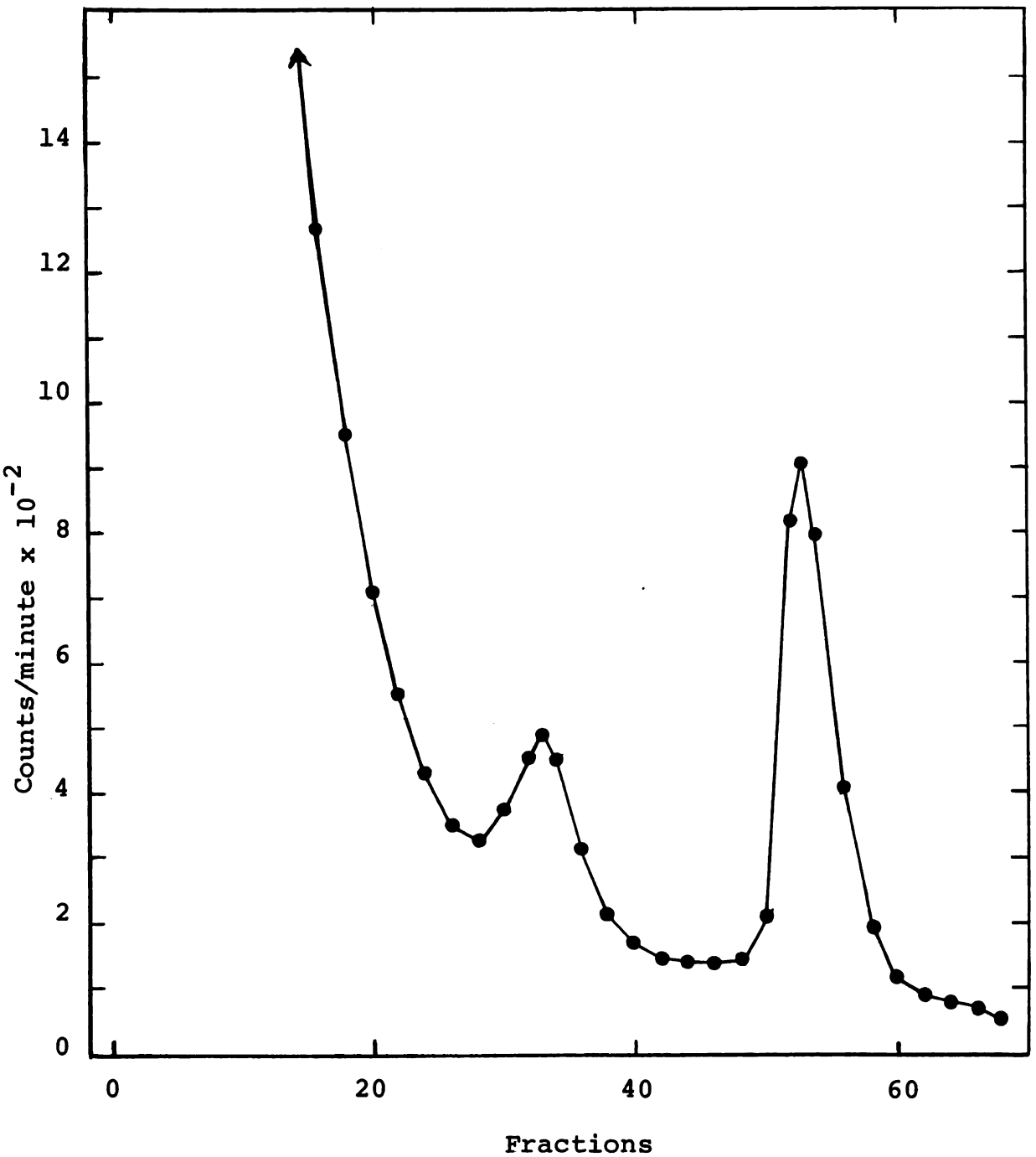

Figure 9.

located in fraction no. 53, and the apex of the smaller peak in fraction no. 33.

Figure 10A depicts the fractions collected from a cesium chloride-ethidium bromide gradient of a lysate of N. gonorrhoeae strain 2686 (colony types 1 and 3). The lysate had been treated with RNAse. The uptake efficiency of the culture was approximately 34.8%. A large peak is evident; its apex is located in fraction no. 56. The band represented by this peak was observed under ultraviolet light prior to collection of the fractions. The band was located approximately 33 mm from the bottom of the polyallomer tube, and was approximately 1.5 mm in width. The data for fractions no. 29 to 39 are missing from figure 10A because these fractions were pooled, dialyzed and layered onto an alkaline sucrose gradient.

Figure 10B depicts the fractions collected from a cesium chloride-ethidium bromide gradient of a lysate of N. gonorrhoeae strain 2686 (colony type 3). The lysate had been treated with RNAse. The uptake efficiency of the culture was approximately 33.0%. A large peak is evident; its apex is located in fraction no. 55. The band represented by this peak was observed under ultraviolet light prior to collection of the fractions. The band was located approximately 33 mm from the bottom of the polyallomer tube, and was approximately 1.0 mm in width. The data for fractions

Figure 10. Cesium chloride-ethidium bromide gradients of N. gonorrhoeae strain 2686. Each ^{14}C-adenine-labeled lysate of N. gonorrhoeae strain 2686 was added to a cesium chloride-ethidium bromide mixture and centrifuged to equilibrium in a Type 50 rotor for 30 hours at 44,000 rpm and 15 C. The lysates had been treated with RNAse. Most fractions were collected onto filter paper squares, washed in TCA, ethanol and ether, and counted for radioactivity. Fractions no. 29 through 39 were pooled, dialyzed and layered onto alkaline sucrose gradients. (A) Colony types 1 and 3. (B) Colony type 3.

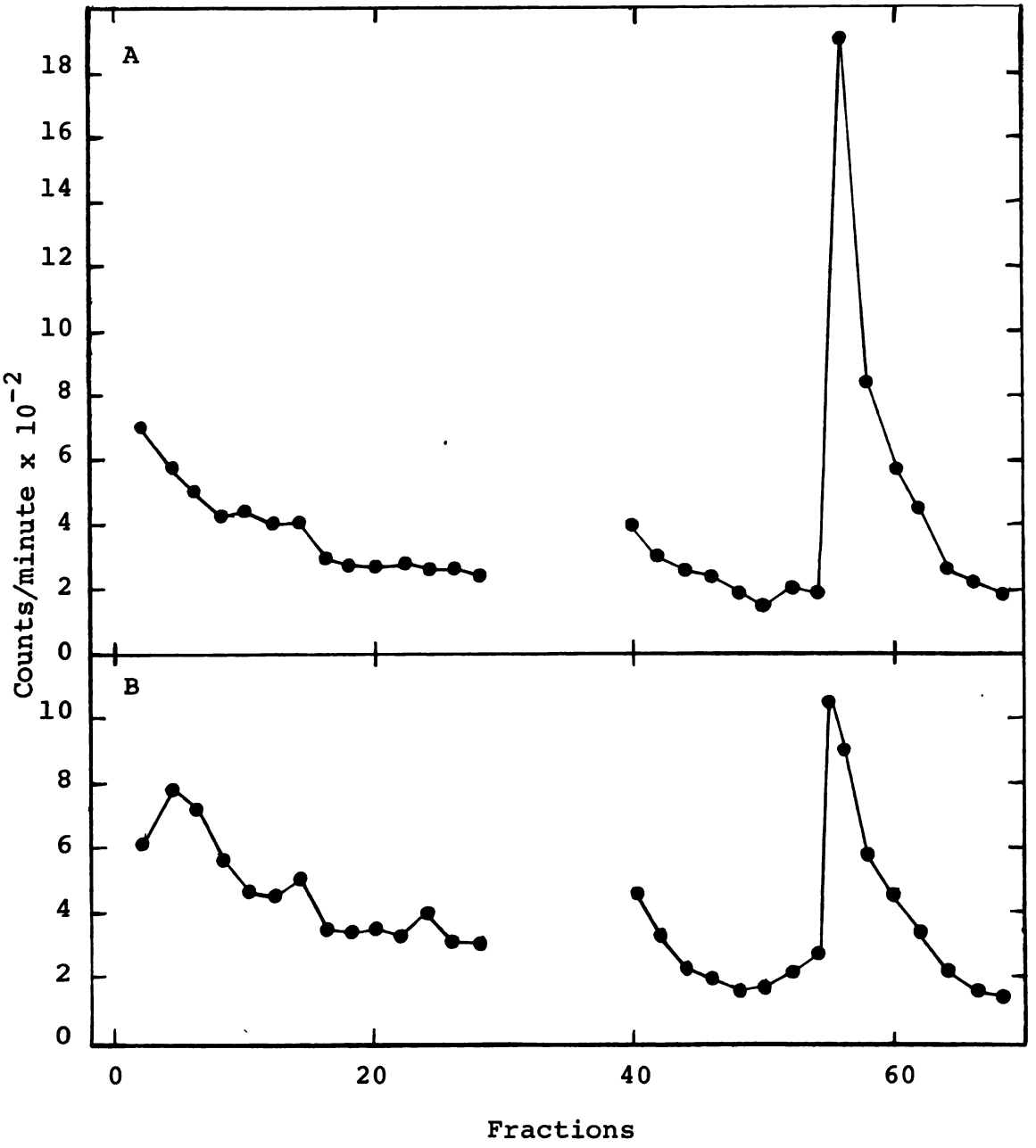
A
B
18
16
14
12
10
8
6
4
2
0
10
8
6
4
2
0
0
20
40
60
Counts/minute x 10^{-2}
Fractions

Figure 10.

no. 29 to 39 are missing from figure 10B because these fractions were pooled, dialyzed and layered onto an alkaline sucrose gradient.

Alkaline Sucrose Gradients: Pooled Plasmid Fractions

Figure 11 depicts the fractions collected from alkaline sucrose gradients of the pooled and dialyzed cesium chloride-ethidium bromide fractions missing from Figure 10.

Figure 11A represents the pooled and dialyzed fractions of N. gonorrhoeae strain 2686 colony types 1 and 3 (Figure 10A). Two peaks are evident.

Figure 11B represents the pooled and dialyzed fractions of N. gonorrhoeae strain 2686 colony type 3 (Figure 10B). Two peaks are evident. These peaks are in the same relative positions as the peaks shown in Figure 11A.

Figure 11. Alkaline sucrose gradients: pooled plasmid fractions. Five-tenths ml of the pooled and dialyzed N. gonorrhoeae strain 2686 fractions from a cesium chloride-ethidium bromide gradient were layered onto a 5.2-ml, 20 to 31% (w/v) linear alkaline sucrose gradient and centrifuged in a SW50L rotor for 90 minutes at 50,000 rpm and 15 C. Fractions were collected onto filter paper squares, washed in TCA, ethanol and ether, and counted for radioactivity. (A) Colony types 1 and 3. (B) Colony type 3.

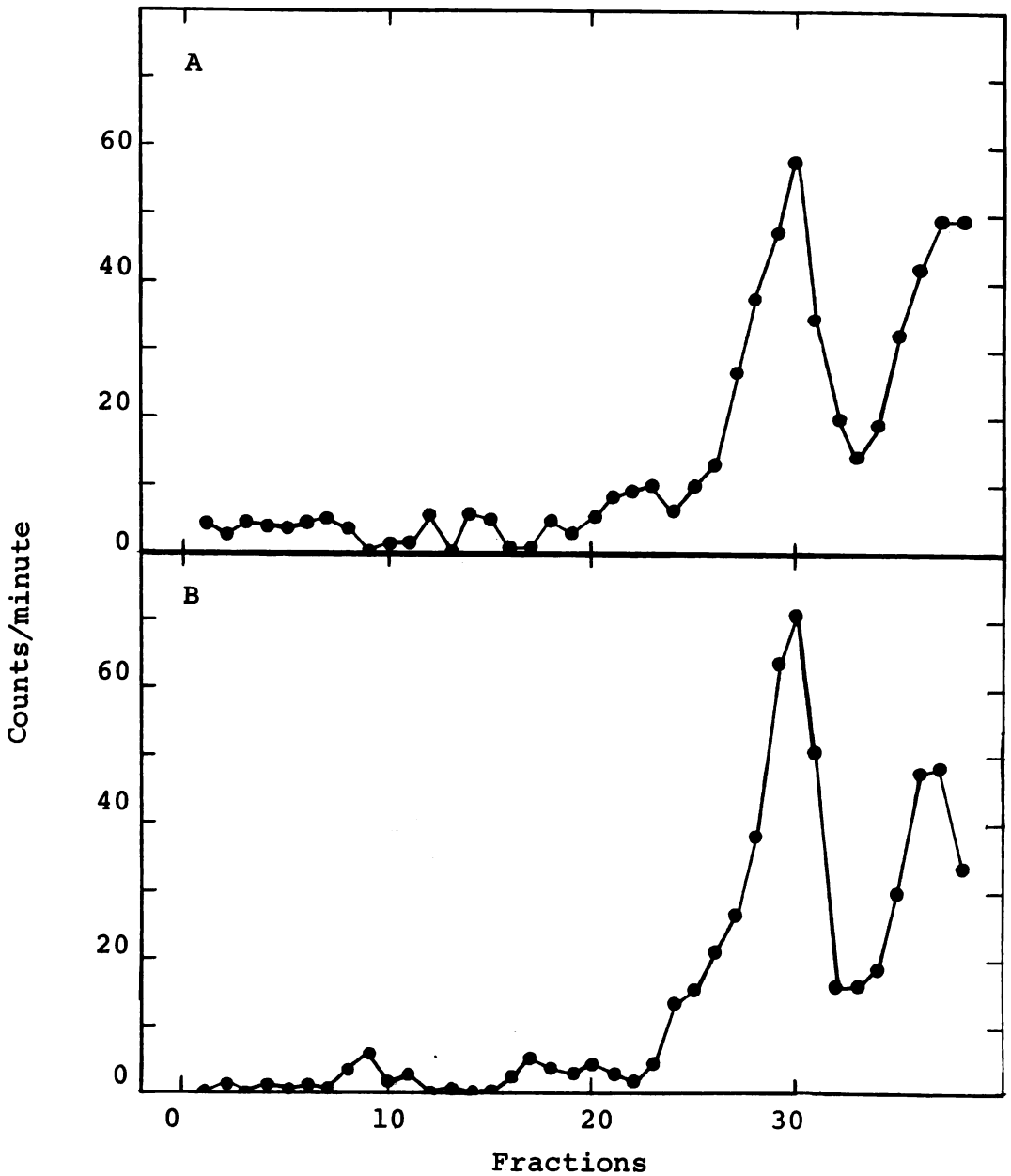

Figure 11.

DISCUSSION

Biphasic Flask System Cultures and Radioactive Labeling

The BFS developed for culturing N. gonorrhoeae yields relatively high cell concentrations in relatively short periods of time. Cell concentrations achieved are higher than those reported by Kenny et al. (35), who utilized a 24-hour incubation period, but are lower than those reported by Gerhardt and Hedén (20), who utilized incubation periods as long as 72 hours. Our estimates of cell concentrations are based upon viable cell counts whereas those of Kenny et al. (35) were based upon Coulter Counter data, and those of Gerhardt and Hedén (20) were based upon turbidity.

Transfer of an aliquot of an early log phase BFS culture into prewarmed "labeling broth" is an adequate method of labeling the gonococcal nucleic acids. Inefficient uptake of ^{3}H-thymidine by N. gonorrhoeae may indicate that gonococci lack the enzymes necessary for incorporation of exogenous thymidine, as has been reported for N. meningitidis (30). Uptake efficiency for ^{14}C-adenine is considerably less than that reported by Kingbury and Duncan

(38) for N. meningitidis, which may indicate that N. gonorrhoeae can not incorporate exogenous adenine as efficiently as N. meningitidis. Uptake efficiencies are probably influenced by such factors as inoculum size, length of the lag phase in the BFS, the number of doublings that occur in the "labeling broth", and permeability differences between strains. Although the final cpm are relatively low, sufficient counts are present to locate the nucleic acid bands in the gradients.

Curing Experiments and Resistance to Antimicrobial Agents

The curing experiment results are contradictory and, therefore, do not provide any information regarding R factors in N. gonorrhoeae. Inconsistancy in the extent to which the clumped gonococci are dispersed probably accounts in part for the variable results. Strains utilized in these experiments cannot tolerate ethidium bromide concentrations as high as those reported for staphylococci or enterobacteria (6), and are greatly inhibited by the concentration that kills genetically competent and incompetent strains of N. meningitidis (27). Gonococci are greatly inhibited by the concentration of acridine orange used to eliminate F^+ from E. coli (23). The concentration of SDS used by Sonstein and Baldwin (73) to eliminate penicillinase plasmids from Staphylococcus aureus causes a 62% inhibition of gonococcus growth.

Most of the freshly-isolated strains of N. gonorrhoeae are resistant to relatively low levels of at least one antibiotic agent. Many are multiply resistant. Results of Hg^{++} ion sensitivity tests indicate that gonococci are as tolerant of Hg^{++} ions as E. coli (39), but slightly less tolerant than S. aureus (50).

Alkaline Sucrose Gradients: Bacterial Lysates

Sheared chromosomal DNA is completely denatured at pH 12.0 and does not sediment very far in a 20 to 31% (w/v) sucrose gradient. Circular, covalently closed plasmid DNA molecules are more resistant to alkali denaturation and sediment more rapidly than sheared, denatured chromosomal DNA.

Each of the three graphs in Figure 7 contain one large peak and two smaller peaks. Each of the large peaks probably represents sheared, denatured chromosomal DNA. The two small peaks in Figure 7A probably represent the two cryptic plasmids of S. pullorum MS83 (77). The small peaks in Figures 7B and 7C may represent N. gonorrhoeae strain 2686 plasmid DNA: either distinct plasmids or different forms of the same plasmid. For each of these three gradients, the total number of cpm recovered represents less than 10% of the estimated total cpm added to the gradient. Unrecovered cpm may represent non-TCA-precipitable fragments of hydrolyzed RNA and unincorporated molecules of ^{14}C-adenine.

Although it was anticipated that RNA would be completely hydrolyzed in the pH 12.0 alkaline sucrose gradients, time did not permit this to be experimentally proven. Since biochemical analyses were not performed on the alkaline sucrose gradient components represented by the peaks, definite conclusions regarding their nucleic acid content cannot be drawn. Peaks obtained from the RNAse-treated lysates are less likely to represent labeled RNA. Bovine pancreatic RNAse was utilized to treat the bacterial lysates. This enzyme is an endonuclease that specifically hydrolyzes only those β linkages of RNA where the ∝ linkages connect phosphoric acid with pyrimidine nucleotides (42). The end products of such RNAse hydrolysis are pyrimidine-containing nucleoside 3'-phosphates and oligonucleotides terminating in a pyrimidine nucleotide with a 3'-phosphate group (42). These end products may then be further hydrolyzed during the 90-minute centrifugation in the pH 12.0 gradient, but the extent of hydrolysis is unknown.

Dye-Buoyant Density Gradients

The cesium chloride-ethidium bromide gradient data of N. gonorrhoeae strain 2686 and S. pullorum are very similar, in that all gradients yield a relatively large peak between fractions no. 50 and 60 and a smaller peak between fractions no. 30 and 40.

The S. pullorum data correlates well with the data reported by Olsen (54), Olsen and Schoenhard (55) and Stiffler (77), who utilized ^{3}H-thymidine-labeled S. pullorum lysates. Therefore, each of the large peaks between fractions 50 and 60 is thought to represent chromosomal DNA, and each of the smaller peaks between fractions no. 30 and 40 is thought to represent plasmid DNA.

The smaller peak in Figure 9, with its apex in fraction no. 33, constitutes the first known preliminary physical evidence of closed circular DNA in N. gonorrhoeae. Such closed circular DNA may be peculiar to strain 2686.

The high counts at the bottom of the gradient depicted in Figure 9 probably represent single-stranded RNA, whose buoyant density is greater than either chromosomal or plasmid DNA. The "plasmid peak" is made artificially taller by its location on the "RNA shoulder." An RNAse treatment reduces, but does not totally eliminate the counts at the bottom of the N. gonorrhoeae gradients (Figure 10). Treatment with RNAse has no apparent effect upon either the "plasmid" or "chromosome" peak.

The reason for a complete lack of RNA counts at the bottom of the S. pullorum gradients (e.g., Figure 8) is unknown. It is possible that the S. pullorum RNA firmly pellets near the bottom of the tube, and is not drawn off as the fractions are collected. N. gonorrhoeae RNA may pellet less firmly. A very high level of RNAse activity in

S. pullorum is another possible explanation, although it is unlikely that the activity is high enough to destroy all of the labeled RNA.

Alkaline Sucrose Gradients: Pooled Plasmid Fractions

The pooled N. gonorrhoeae strain 2686 cesium chloride-ethidium bromide fractions yield two distinct peaks when centrifuged in an alkaline sucrose gradient (Figure 11). Both of these peaks may represent DNA bands, but the possibility that one or both of the bands contain RNA cannot be ruled out completely. Time did not permit the performance of experiments designed to determine the biochemical nature of the material present in the bands.

The more rapidly-sedimenting band is thought to represent plasmid DNA for the following reasons:

1. The pooled fractions are obtained from the region of a cesium chloride-ethidium bromide gradient where S. pullorum MS83 plasmids are known to band.

2. The pooled fractions are obtained from regions of the N. gonorrhoeae strain 2686 cesium chloride-ethidium bromide gradients which give evidence of a small peak.

3. Treatment with RNAse reduces the quantity of RNA present in the cesium chloride-ethidium bromide gradients.

4. The pH of the alkaline sucrose gradients probably causes some hydrolysis of any RNA present in the pooled fractions.

5. Only TCA-precipitable nucleic acid fragments remain on the filter paper squares at the conclusion of the washing procedure.

The peak at the top of the gradient may represent 1) a different molecular configuration of the same plasmid, 2) a second plasmid, 3) TCA-precipitable RNA fragments, 4) a combination of (2) and (3), or 5) a combination of (1) and (3). The final possibility is favored.

Extrachromosomal DNA in N. gonorrhoeae Strain 2686

The frequency with which type 1 colonies give rise to type 3 colonies suggests that the conversion of the piliated type 1 variant of N. gonorrhoeae strain 2686 to the nonpiliated colony type 3 variant represents the spontaneous loss of a plasmid carrying the markers determining colonial morphology and the expression of pili. The gradient data does not support this hypothesis.

Preliminary physical evidence of extrachromosomal DNA in the nonpiliated colony type 3 variant as well as the mixture of piliated type 1 and nonpiliated type 3 variants suggests two possibilities:

1. Type 1 colony-producing cells contain the same complement of extrachromosomal DNA that type 3 colony-producing cells do. In this case, the plasmid may have no connection with either colonial morphology or the expression of pili.

2. All of the satellite band DNA in the N. gonorrhoeae strain 2686 cesium chloride-ethidium bromide gradients comes from the type 3 colony-producing cells.

The latter hypothesis is favored, and is expanded as a possible explanation for certain phenomena observed during the course of this investigation. The plasmid harbored by strain 2686 is episomic. Only when it is integrated into the host chromosome is the cell capable of expressing pili. Such a cell is genetically unstable during in vitro cultivation, and will give rise to a type 1 colony. A type 1 colony represents a mixed population of piliated and nonpiliated cells. The nonpiliated cells of the type 1 colony harbor the plasmid in an autonomous state or have lost the plasmid completely. A cell harboring the plasmid in its autonomous state will give rise to a type 3 colony. The type 3 colony thus represents a population of cells, each of which harbors an episomic plasmid in its autonomous state. Reintegration of the plasmid into the host chromosome is apparently a very rare event since subcultures of type 3 colonies are stable.

The expanded hypothesis is similar to that proposed by Jyssum and Lie (29), Jyssum and Jyssum (28) and Jyssum (26, 27) to explain genetic competence in N. meningitidis. If gonococcal pili play some role in transformation, the above hypothesis could serve to explain Sparling's (74)

observation that type 1 gonococcus colonies give transformation frequencies which are 2 x 10^4 times greater than those of colony type 3.

Numerous questions were generated during this investigation and, unfortunately, time did not permit the experimentation considered necessary to find answers to these questions. Hopefully, future investigations will include:

1. Experiments designed to confirm that the "plasmid" peaks obtained actually represent circular, covalently closed double-stranded DNA molecules.

2. Cosedimentation experiments designed to characterize the plasmid molecules.

3. Genetic experiments designed to determine the markers carried by the plasmid molecules.

Such future investigations should contribute significantly to the limited body of knowledge concerning the genetics of N. gonorrhoeae.

SUMMARY

The ultimate objective of this investigation was an examination of multiple antibiotic resistant and piliated strains of Neisseria gonorrhoeae for evidence of extrachromosomal DNA. Before this objective could be realized, it was necessary to develop culturing, labeling and lysing techniques.

A biphasic flask system composed of dextrose-starch solid medium overlaid with phosphate-buffered liquid medium was developed to obtain liquid cultures of gonococci for temporary preservation, plasmid-curing and radioactive labeling purposes. Concentrations on the order of 10^8 to 10^9 cells/ml were obtained in an 11-hour incubation period.

Transfer of an aliquot of an early log phase biphasic flask system culture into prewarmed "labeling broth" proved to be an adequate method of labeling gonococcal nucleic acids with ^{14}C-adenine. Although uptake efficiencies averaged only approximately 29%, sufficient incorporation was achieved to locate the nucleic acid bands in the gradients.

The lysis procedure of Clewell and Helinski (14) was modified by substituting Triton X-100 for Brij 58, increasing the sodium deoxycholate concentration and freezing

the cells in sucrose solution before lysing. These modifications resulted in relatively clear and viscous N. gonorrhoeae and S. pullorum lysates with no apparent damage to circular, covalently closed DNA molecules.

Dye-buoyant density gradient techniques previously utilized for S. pullorum research were successfully employed to obtain the first known preliminary physical evidence for plasmids in N. gonorrhoeae. A hypothesis was proposed to explain the correlation between gonococcal pili and plasmids.

Additional aspects of the investigation concerned plasmid-curing and electron microscopy. Plasmid-curing experiments utilizing ethidium bromide, acridine orange, and SDS yielded contradictory data. The electron micrographs confirmed the presence of pili on cells from gonococcus colony types 1 and 2.

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LITERATURE CITED

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