DIALYSIS CULTURE OF NEISSERIA GONORRHOEAE

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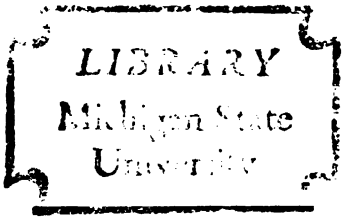

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ABSTRACT

DIALYSIS CULTURE OF *NEISSERIA GONORRHOEAE*

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Neisseria gonorrhoeae strain 2686, colony type T_2, produced good cell yields and maintained above 92% T_2 colony type morphology in a modified Marbrook Chamber. Various methods were used to disperse agglutinated T_2 gonococcal cells during growth in the Marbrook Chamber, but only sonication of samples at 20 watts/second for 2 seconds increased colony forming units/ml as enumerated in viable counts by the surface plating method. Corn starch (0.1%), 1% soluble starch, and 2% bovine serum albumin were compared for adsorbant capacity, with highest yield of colony forming units/ml obtained using 0.1% corn starch and 1% soluble starch. Viable cell yields from the Marbrook Chamber were not as high as those obtained in comparison to the Biphasic Culture System and the Liquid Flask Culture. Finally, gonococcal cells maintained T_2 colony type and the colony forming units/ml increased in a clear menstruum of cysteine phosphate buffered saline in both the Marbrook Chamber and Biphasic Culture System. However, statistical analysis (P=0.5) showed no significant difference in growth of *Neisseria gonorrhoeae* in any of the methods employed.

Growth was not obtained when *Neisseria gonorrhoeae* strain 2686, colony type T_2, was added to the Prosthetic Hemodialysis Culture Unit in an attempt to propagate the gonococcal cells. *Neisseria gonorrhoeae* was found to be sensitive to heparin, the anticoagulant in the goat, and displayed small size and reduced growth in agar plate sensitivity tests using 125 U, 250 U, and 500 U/ml of heparin. Decreased viability was found in heparinized goat serum but, when incubated in the Marbrook Chamber in the presence of increasing concentrations of heparin, the colony forming units/ml of gonococci increased. This discrepancy might be due to the fact that the heparin was incorporated in two different phases. Whether it is actually inhibitory to growth of *Neisseria gonorrhoeae* in the Prosthetic Hemodialysis Culture Unit is yet to be determined.

DIALYSIS CULTURE OF *NEISSERIA GONORRHOEAE*

By

Leslie Lynne Ruezinsky

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Dedicated to my husband

Gary

and my mother and Bill

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INTRODUCTION

Although *Neisseria gonorrhoeae* has been recognized as a cause of venereal disease for many centuries, not much has been learned about its pathogenesis. One of the major drawbacks to research has been the absence of an animal model which sufficiently simulates human disease. Penicillin has been used successfully to cure infection but, due to increasing resistance to penicillin and asymptomatic cases of gonorrhea, the disease is still rampant. No effective serological screening test or vaccine has been developed.

Kellogg *et al.* (84) described 4 colony types and demonstrated a relationship between colonial type and virulence for human volunteers. Types 1 and 2 were most often cultured on primary isolation from infectious material and after nonselective transfer they underwent a transition to type 3 and type 4. The colonial types 1 and 2 were virulent for human volunteers (83,84), possessed pili (78,154), showed an increased ability to attach to tissue cells (28,75,152, 178,179,180,182,183) were more competent in transformation studies (30), and were more resistant to phagocytosis by human polymorphonuclear leukocytes (129,166,168,169,190) than types 3 and 4. Types 1 and 2 also revert rapidly to types 3 and 4, and tend to clump in liquid culture (78,84), which makes studying these colonial types difficult.

Virulence of colonial types 1 and 2 has been further studied using chicken embryos (24,26), "practice golf balls" (6), and metal and vinyl coils placed under the skin of small animals (7). The chimpanzee has been the best experimental subject. Infection in this animal resembles the human disease more than infections in other animals used so far but, because of management and expense, its use is usually impractical for most research.

This study examines the use of dialysis as a new method for cultivation of *N. gonorrhoeae*. Previous researchers, using a biphasic culture system (57) consisting of 100 ml of agar overlaid with 25 ml of broth culture medium, had obtained higher yields than with liquid flask cultures. Based on the success of this dialysis model, the dialysis chamber devised by Marbrook (108) was chosen for this study as an *in vitro* culture system.

For *in vivo* study, a new dialysis system called the Prosthetic Hemodialysis Culture Unit of Quarles (130) was tested. This small hemodialysis culture unit was connected to two shunts surgically implanted in the neck of a goat, connecting the carotid artery and the jugular vein.

It was hoped that the *in vitro* studies would provide a new technique for cell production and maintenance of colony type morphology, and that the *in vivo* study would provide a new animal model simulating bacteremic infection in man.

REVIEW OF THE LITERATURE

History of the Gonococcus

There are many suggestive references to gonorrhea in ancient Egyptian, Chinese, Japanese and Biblical writings (163). Galen named the disease in 130 A.D., mistaking it for spermatorrhea (61,142,163). It was not recognized to be of venereal origin until the 13th century when Gulielmus de Salicito attributed the affliction to impurities retained under the male prepuce after contact with unclean females (163). Paracelsus thought it was an early symptom of syphilis in 1530 and John Hunter further confused it with syphilis in 1767. It was not until 1837 that Ricord delineated the two diseases clearly and firmly.

In 1879 Neisser described the gonococcus as the causative agent of purulent urethral and vaginal discharges, and exudates from the eyes of infants with neonatal ophthalmia (61,142,163). Leistikow and Loeffler succeeded in cultivating the organism in 1882 and Hans Gram furthered the general identification with the application of the Gram stain in 1884 (61,142,163). Von Bumm in 1885 isolated pure cultures of gonococci from an infected patient and was able to experimentally transmit the disease in human volunteers (142,163). Before the advent of sulfonamides, treatment was unsuccessful and, since the quick development of resistance to sulfonamides, penicillin

has been the drug of choice. Even with effective and adequate therapy, gonorrhea is still rampant in the U.S. today.

Classification of the Gonococcus

The gonococcus is classified in the family Neisseriaceae under the genus *Neisseria* named for Dr. Albert Neisser. It is characterized as a Gram negative coccus, 0.6-1.0 μm in diameter, occurring singly but most often in pairs with adjacent sides flattened--simulating the shape of a coffee bean. Other characteristics include fastidious and complex nutritional requirements, production of catalase and oxidase, and fermentation of glucose only (135). The gonococcus is also nonmotile, nonsporogenous and requires increased CO_2 tension for adequate growth (142). Other species within the genus include *Neisseria meningitidis, N. sicca, N. subflava, N. flavescens* and *N. mucosa* (135).

Colonial Morphology and Variation

Since 1904 when Lipschutz first reported differences in the colonial morphology of *N. gonorrhoeae*, many other researchers have recorded interesting observations about colonial morphology (21), but it was not until 1963 that the gonococcus was classified by Kellogg *et al.* (84) into four distinct colony types using translucent media under specific growth conditions. With selective transfer these distinct colony types can be perpetuated and are best visualized with the use of diffused, angled transmitted light, after 18 to 24 hrs of incubation at 36°C with increased CO_2 tension (84). The different colony types are designated T_1, T_2, T_3, T_4 and, more

recently reported, T_5 (78). Many other researchers have confirmed and added to these observations.

T_1 colonies are small, about 0.5 mm in diameter, dark gold to black in color, translucent, convex with a soft but distinct colonial edge (78,83,84,95,137). They are also slightly viscid in consistency, easily emulsified and agglutinate when suspended in saline (78,84). T_2 colonies are slightly smaller than T_1 colonies (0.4 mm in diameter), dark gold to black, translucent, and convex like T_1 colonies. T_2 is differentiated from T_1 by its slight internal granularity, a very definite clear-cut colonial edge with a sharp increase in height at the periphery of the colony, a friable consistency and very shiny, refractile surface. Saline suspensions of T_2 gonococcal colonies are very "rough" with a greater increase in agglutination than T_1 colonies (83,95,137).

T_3 colonies are larger in size than T_1 and T_2 (1.0-1.2 mm in diameter), light brown in color, translucent, flat and have an internal granularity. The colony consistency is viscid and T_3 gonococci are slightly agglutinated in saline. T_4 colonies are the same size and elevation as T_3 colonies but are colorless, amorphous, viscid, and form a smooth suspension in saline (78,83,84,95,137).

Jephcott and Reyn first reported T_5 colonies in 1971 as very dark brown, coarsely granular colonies around the size of T_3 and T_4. T_5 colonies are very shiny like T_2 but with coarse irregular edges and a concentric ring pattern on the surface (78,137). Brown *et al.* (21) presented color photographs of all 5 colony types which aid in identification. A summary of colonial characteristics is presented in Table 1.

Table 1. Colonial characteristics of *Neisseria gonorrhoeae*

Colony type	Size	Color	Elevation	Edges	Consistency	Macroscopic surface texture
T_1	0.5 mm	dark gold - black	convex	soft, entire	slightly	amorphous
T_2	0.4 mm	dark gold - black	convex	slightly crenated, very defined	friable	slight internal granularity; shiny surface
T_3	1.0-1.2 mm	light brown	flat	soft, entire	viscid	internal granularity
T_4	1.0-1.2 mm	colorless	flat	soft, entire	viscid	amorphous
T_5	1.0-1.2 mm	dark brown	slightly convex	coarse, irregular	not reported	coarse, internal granularity; concentric ring pattern

Selective subculture of the different colony types on solid media must be employed to cultivate successively each colony type, especially the T_1 and T_2 colonies because of the reversion of T_1 to T_4, and T_2 to T_3 (78) after 30 h of incubation and under conditions of non-selective transfer. Stabilization of T_1 and T_2 colonies has not yet been attained with available media since they seem to be more sensitive to changes in their physical and chemical environment than T_3 and T_4 (84). Back reversion of T_4 colonies to T_1, T_2 and T_3 colonies and interconversions between T_3 and T_4 have been reported (78,83,172). T_2 colonies seem to be the least stable, and freshly isolated cultures appear to be less stable than those that have been repeatedly subcultured (78).

The gonococcus has also been observed to assume morphological types other than those most regularly reported. Jephcott and Reyn reported granular forms that did not resemble previously documented types (78). Kellogg *et al.* (83) observed an intermediate colony type--T_{21}--which has an increased surface roughness and variations in colonial border. Several investigators have described dark and light color variations within colony types T_1 and T_2 (21,38,142,146). Diena *et al.* (38) studied the light and dark variations of colony types T_1 and T_2 in greater depth and found that the darker types (T_1 and T_2) reverted to only T_3 while the lighter types (T_{1a} and T_{2a}) generated only T_4. They could see no difference in the morphology of the lighter and darker types by light or electron microscopy. Sparling (146) also reported small, rough, granular type colonies which correspond to Kellogg's T_{21}, but he raised the possibility

that this variation could have been due to excessive plate moisture, a phenomenon which Kellogg (84) also reported. No distinct capsule has been found on any of the five colony types (83).

Colonial Morphology and Virulence

Much work has been done to correlate colonial morphology with virulence. Kellogg and his associates first demonstrated this in 1963 (84) and in studies in 1968 using human volunteers (83). T_1 and T_2 colony types were found to be virulent producing moderate amounts of purulent exudate and other clinical symptoms of gonorrhea. Inoculation with T_3 and T_4 colonies resulted in either a watery discharge or none at all, and no infection after 24 hours (83). This correlation of virulence has been reported experimentally with chicken embryos (24) and in observations on the morphology of freshly isolated cultures (93,146) of which 99% of the cultures contained T_1, T_2 or both colony types (146). The presence of pili, only on T_1 and T_2 colonies (see Ultrastructure), increased resistance of T_1 and T_2 colonies to phagocytosis (see Phagocytosis and Virulence), increased competence for genetic transformation (see Metabolism of the Gonococcus and Genetics of the Gonococcus), and increased resistance to complement-mediated serum bactericidal reaction of T_1 and T_2 colony types (see Phagocytosis and Virulence) also correlate colonial morphology with virulence.

L Forms of the Gonococcus

L forms of *Neisseria gonorrhoeae* were first reported in 1964 by Dienes *et al.* (40), after they isolated "large bodies" from

original cultures using a special medium and growth conditions. After penicillin or glycine were added to the cultures, all gonococci formed "large bodies." These "large bodies" autolyzed and produced a secondary growth of cocci. Roberts (138) also isolated L forms of *N. gonorrhoeae* using penicillin gradient plates and found two morphological types that he classified as L forms, i.e., large colonies with little or no periphery and small colonies with well defined peripheries. These L forms were resistant to 4 of 8 antibiotics that inhibit cell wall synthesis, and reverted to bacterial forms when transferred to penicillin-free media. It was found in one study that 4.5% of gonorrhea cases were detected only by isolation on L form media (59), and another source implicated L forms of *N. gonorrhoeae* in penicillin failures (11).

Ultrastructure

Using the electron microscope it has been found that the cell wall and cytoplasmic membrane of *Neisseria gonorrhoeae* is structurally and biochemically similar in composition to other Gram negative membranes (80). The outer membrane was found to contain three proteins with molecular weights of 34,500, 22,000 and 11,500 daltons. The enzymes isolated from the cell wall were succinic dehydrogenase, NADH oxidase and D-lactate dehydrogenase. The cell wall consisted of 3 layers 80 angstroms (Å) thick, with 2 dense zones each 30 Å thick, and a ligher central zone (53,151). The cell membrane was approximately 95-100 Å wide and of the unit membrane type with a double-line profile (53,79). The cell wall-cytoplasmic membrane interface was convoluted (53,123). These convolutions gave the

surface of the gonococcus a wrinkled appearance. The outer surface also appeared to be pebbly and pitted in texture, with evaginations or blebs extending from the cell which seemed to originate partially through the cell wall outer membrane (95,151). The pits or "pores" were about 80-100 Å in diameter and appeared numerously and randomly over the surface of the gonococcus (53,154). The blebs or small spherical structures ranged in diameter from 0.2-0.6 μm and were found on the bacterial surface, on the pili or completely free from the gonococci. The blebs were least numerous on the T_4 colony types (95). Pili or fimbria were found only on colonial types T_1 and T_2, and will be discussed below.

The gonococcal cytoplasm was of uniform density with striated plates occasionally observed which appeared to be of a fibrous nature instead of crystalline as found in several other bacterial species (86). Kellogg (86), with the use of a Ribi cell fractionator, isolated small structures resembling transparent balls with an average diameter of 1.00 $\pm$ 0.05 μm, containing granules and having a pliable membrane-like surface structure, from the cytoplasm of T_1 gonococcal cells. During cell division there was septum formation preceded by a loop of membrane, with mesosomes visible at opposite poles of the cell. They may have functioned by holding the separated chromosomes away from the plane of division (53). The completion of cell wall formation was very slow and this resulted in delay in the separation of already divided cells and thus diplococcal formation. Cell wall-cytoplasmic membrane junctions have also been observed (53,86) and it appeared that the cell wall was

thinnest at these points and this could have been a point of peripheral cell wall synthesis.

Recently, colonial types T_1 and T_2 were found to exhibit pili or fimbrae radiating from their surfaces and joining adjacent cells. The pili have only been found on T_1 and T_2 colonial types of *N. gonorrhoeae*, and on some of the nonpathogenic species of *Neisseria* (79,154,190). Pili are a class of bacterial surface filaments which are smaller in diameter than flagella, do not have the amplitude, wavelength or sinuous form of flagella, have no motility function and no terminal attachment apparatus (154,173,190). They consist of hollow tubes of polymerized protein molecules which extend through the cell wall-membrane barrier (15) and can be found on other organisms including *Pseudomonas, Klebsiella, Vibrio, Acinetobacter, Streptococcus Gp. A,* and *Corynebacterium renale* (173,190).

Pili were first described as fibrils individually measuring 80-85 Å in diameter, usually found in large bundles, of uniform thickness and most abundant on T_2 gonococci but also found associated with T_1 gonococci. They tended to form lateral aggregates, vary in length from 0.5 to 2 μ and also have a faint periodicity of approximately 50 Å (154). The number of pili per cell varied from 2 to 40 and reached maximum numbers when the gonococcus was cultured on solid agar and then incubated for a short time in liquid media (173). They were composed of protein and were antigenic (25,129, 142,173,190), and it was suggested that they could contribute to virulence of the gonococcus. The presence of pili has been correlated with resistance to phagocytosis by human polymorphonuclear leukocytes

and increased ability of the gonococcus to attach to human amnion, epithelial, fibroblast and human sperm cells (see Pili and Virulence).

Another important surface component of the gonococcus was the zones of focal adhesion between the cell walls of colonial types T_1, T_2 and T_3. Cells of T_4 had few zones of adhesions between the cells. The zones stained with lanthanum nitrate measured 20 Å in width. They resembled gap junctions in animal systems but did not display the same type of membrane modifications (154). They did not seem to be related to planes of division because there were many zones, and zones were found between both young and old gonococci. It was suspected that they represented regions of stickiness and helped contribute to the clumping of the gonococci of cell types T_1, T_2 and T_3 in saline suspensions (142,154).

Finally a recent report by Grimble and Armetige (65) described larger appendages with a diameter of 20-30 μm, varying in number per cell. They had a terminal bulb which was seen on pili also, and had beading along the length of the appendages. These large appendages also made contact with other cells.

Metabolism of the Gonococcus

An early study of the composition of the gonococcus by chemical analysis yielded 5-9% total carbohydrate, 10-14% total lipid, 1% sulfur, 13-18% volatile and nonvolatile substances and 12.4% total nitrogen by weight. Nucleoproteins composed 60-65% of the cell by weight (148).

The metabolism of *Neisseria gonorrhoeae* has so far been incompletely characterized. Glucose was the only sugar metabolized and

pyruvate and lactate were other carbohydrates utilized (119). Early studies revealed that glyceric and alpha-hydroxybuteric acids were moderately oxidized (12). Glucose was found to be metabolized stricly by aerobic mechanisms in two stages. The first stage utilized a combination of Entner-Doudoroff, and pentose phosphate pathways during active culture growth (119). Enzyme analysis confirmed the presence of all enzymes of the Entner-Doudoroff, pentose phosphate and Embden-Meyerhof-Parnas pathways, although the Embden-Meyerhof-Parnas pathway did not appear to function in *N. gonorrhoeae* under conditions studied so far (71,119). It was indicated that the Entner-Doudoroff pathway was the major route of metabolism utilizing 80-87% of the glucose and the pentose phosphate pathway participated to a lesser extent, utilizing 13-20% under normal growth conditions. Acetate and CO_2 were the end products of the first stage of glucose metabolism (16,119). The second stage of glucose metabolism was the oxidation of acetate by the tricarboxylic acid cycle following depletion of glucose (140). Since *N. gonorrhoeae* has been shown not to utilize the Embden-Meyerhof-Parnas pathway of glucose catabolism, either aerobically or anaerobically, the expression "fermentation of glucose" should probably be replaced by "production of acid from glucose" (12,119).

Neisseria gonorrhoeae like all other *Neisseria* contained a cyanide sensitive, aerobic cysteine oxidase (159) and the presence of this cytochrome oxidase enabled the organism to rapidly oxidize dimethyl- or tetramethyl-paraphenylene diamine (oxidase reaction). Most aerobic and facultatively aerobic bacteria contain a cytochrome

system which acts as an electron carrier in aerobic respiration (61,147).

The four major cellular fatty acids present in *N. gonorrhoeae* are palmitic, palmitoleic, B-hydroxylauric and lauric acids (121). No hydroxy acids were found in *N. gonorrhoeae* (17). *Neisseria gonorrhoeae* was also found to produce free fatty acids and phospholipids that were self-inhibitory and in studies utilizing methyl esters of the major free fatty acids and the major phospholipids, it was found that the probable degradation products of the major phospholipid component of *N. gonorrhoeae* (phosphatidylethanolamine) were inhibitory to most strains. The inhibitory effect of these degradation products, monoacyl phosphatidylethanolamine and long chain free fatty acids, could have been surfactant in nature but they could also have inhibited substrate transport into the cell or prevented phosphorylation of thiamine, an essential step for the entry of fatty acids into the tricarboxylic acid cycle (142,176). The inhibition of growth by free fatty acids and phospholipids will be discussed further under Identification and Cultivation.

Genetics of the Gonococcus

Gonococci have about 1.5×10^6 nucleotide pairs, i.e., about 40% of the DNA found in *Escherichia coli* (92). Genetic transformation has been found to occur in *N. gonorrhoeae* and competence was found to be greater in T_1 and T_2 colony types (1%) than in T_3 and T_4 (0.00005%). Competence was maintained throughout the growth cycle but seemed maximal in lag and early log phases of growth. There was the possibility that pili played a role in uptake of DNA

and could have accounted for the greater competence of T_1 and T_2 colony types (30). Transformation studies on gonococci have been used to verify the hereditary basis of gonococcal auxotypes (see Identification and Cultivation) and establish that many different mutations existed in potentially virulent gonococci. Transformation to prototropy in these studies supported previous findings that T_3 and T_4 colonies produce little or no transformation (30,145). Intra- and interspecies transformation was found to occur in several other species of *Neisseria* (31), and recently Wood *et al.* (191) was able to transform a leucine requiring, rifampin-sensitive strain of *N. gonorrhoeae* to a leucine-nonrequiring, rifampin-resistant phenotype using DNA from *N. meningitidis*.

Plasmids have been isolated from *N. gonorrhoeae* (43), but so far no bacteriophage has been recovered (176), and recent work reporting the isolation of self-inhibitory free fatty acids by *N. gonorrhoeae* strongly suggests that the production of bacteriocins as previously reported (49) is questionable (176). It has been suggested that gonococcal pili and colonial type may be determined by plasmid genes (30,142), but in recent research on the characterization of gonococcal plasmids, plasmids were also isolated from T_3 and T_4 cells (113) and therefore plasmids do not appear to be related to the *in vitro* colonial variation of gonococci associated with loss of virulence. This report also measured the average size of gonococcal plasmids as being 2.37×10^6 daltons with a G+C content of 0.50, and 24-32 plasmid copies per chromosome equivalent. Conjugation can be discarded as a theory of the mechanism of genetic

transfer because maximum frequency of recombination was obtained when recipients were transformed with the supernatant of a sterile broth culture (141). It was thought earlier that a common genetic basis existed for resistance to multiple drugs which could be due either to plasmids of the "R factor" type, mutational alteration of cell membrane permeability causing decreased uptake of drug, or antibiotic-inactivation enzymes (107,109,139,158). Studies using multiple drug resistance have shown a single step mutational loss of 6 drugs and a reacquisition by spontaneous mutation (107). Cotransformation frequencies have led to the genetic mapping of genes for resistance to rifampin, streptomycin, tetracycline, chloramphenicol and spectinomycin in that order (140). Using this multiple resistance it was found that plasmids were not likely the basis of transfer of genetic information due to resistance to "curing" with ethidium bromide (107) but no evidence has proved that extracellular transforming DNA is plasmid or chromosomal in origin (141). It also has been shown that mutation to high level resistance to either streptomycin or spectinomycin was associated with 30s ribosomal resistance to the drug and it was unlikely that antibiotic-inactivating enzymes were the mechanism of resistance as was the case with other bacteria (106).

Colony Type and Virulence in Human Disease and Animal Models

Little has been learned about the pathogenesis of gonorrhea due to lack of an experimental model which sufficiently simulates the human disease (69). Recently chimpanzees have been successfully infected with gonorrhea and have sexually transmitted the disease.

Neisseria gonorrhoeae has also been cultivated in chicken eggs and in small chambers implanted under the skin of small animals, but these models are limited in their resemblance to human infection (see Identification and Cultivation).

Before work was started with chimpanzees, some experimentation was carried out with human volunteers. This enabled Kellogg *et al.* (84), using an inoculum of approximately 10^{10} bacteria to discover that T_1 and T_2 colonial types were virulent while T_3 and T_4 seemed to be avirulent. It was observed that 90% of primary isolates from infected patients consisted of T_1 colonies and 10% of the isolates yielded T_2 and T_3 colonies. After 69 and 440 selective transfers T_1 and T_2 were found to be virulent, and produced copious amounts of purulent exudate and other symptoms of infection; avirulent T_3 and T_4 resulted in a watery discharge or none at all (83,84). An interesting observation was that when the inoculum was 100% T_1, successive daily isolations showed a change to 90% T_2 and when the inoculum was 100% T_2, isolates changed to 90% T_1. Sparling (146) also confirmed that in 99% of cultures from infected males and females, T_1 or T_2 or both colonial types were isolated, and he also pointed out that there was no correlation between duration of infection or symptoms related to a specific colony type in either males or females. These studies on virulence have been confirmed in the chicken embryo using the chorioallantoic cavity and intravenous routes of inoculation. In studies on the chorioallantoic cavity route of inoculation, T_1 and T_2 colonial types were found to produce infection and contribute to the death of 69% of the embryos as

compared to 12% using T_3 and T_4 (24). With intravenous inoculation, T_1 and T_2 colonial types demonstrated reduced initial clearance from the blood and increased ability to multiply when compared with T_3 and T_4 colony types (26).

Pili and Virulence

One of the properties associated with *N. gonorrhoeae* that may contribute to its pathogenesis is its ability to attach to various types of cells *in vivo* and *in vitro*. It was found that piliated gonococcal cells (T_1 and T_2) have a marked ability to adhere to the epithelial and mucous secreting cells of the human urethra (178,179, 180) and fallopian tube (28,182), sperm (75), erythrocytes (129), amnion cells (152), polymorphonuclear leukocytes (129,166,183), fibroblasts (180), and MK_2, HeLa and WI-38 tissue culture cell lines (172). In studies on the association of *N. gonorrhoeae* and mucosal and epithelial cells of the urethra it appeared that the gonococcus fits into microcontours on the cell surface and then cell processes (microvilli) appear to twist over and attach to the bacteria with the epithelial cell membrane pushing up around the gonococci (178, 179). It has been postulated that attachment to human urethral mucosa helps prevent dislocation of the gonococci during micturition (180). Human fallopian tubes maintained in perfusion and organ culture systems have revealed that the microvilli from the mucosal surface wrap around the gonococcal cells, and pili from the gonococci course among the microvilli and run over the epithelial cell membrane (182).

Ciliated epithelial cells showed no attachment of gonococci (28,182). Studies using amnion cell culture also showed pilus-amnion connections which also involved microvilli from the amnion cell (152). Zones of adhesion between the gonococcal cell and the amnion cell were also seen in this study, which could have been an event which happened after contact had been made by pili. Piliated gonococci have also been found to agglutinate various mammalian and chicken erythrocytes, and depiliated T_1 gonococci failed to attach to epithelial cells and agglutinate erythrocytes (129). Antipili antibody also inhibited attachment of piliated gonococci to human epithelial cells, erythrocytes and sperm cells (75,129). Some nonpiliated species have been found to attach to sperm and human oviduct but not to as great an extent as piliated gonococci (75,152). Nonpathogenic *Neisseria* also can attach to other cells by pili, so although attachment of pili to mucosal surfaces may promote colonization, this suggested another mechanism(s) must be involved in pathogenesis (104,180,182). It has been posulated that the attachment of gonococci to the mucosal and epithelial cells is an essential prerequisite for invasion of deeper tissue and that just attachment and growth without invasion of deeper tissue could be an explanation of the carrier state (178).

Phagocytosis and Virulence

Earlier research revealed that increased resistance of piliated *N. gonorrhoeae* to phagocytosis by polymorphonuclear leukocytes could be another property or mechanism contributing to the virulence of *N. gonorrhoeae* (129,166,168,169,190). Heat-killed T_1 gonococci

showed more resistance to phagocytosis than T_4 colonies (124,129,169), while mechanically and enzymatically depiliated T_1 gonococci were found to have reduced resistance to phagocytosis (129,169). Gonococcal pili were also found to be sensitive to trypsin, and inhibitors of protein synthesis were found to inhibit production of pili suggesting that a protein component(s) of pili is an important determinant of antiphagocytic properties of the gonococci (129). Recently Swanson *et al.* (156) reported that he found a strain of T_4 gonococci that had the same or greater attachment-ingestion properties than T_2 gonococci and therefore suggested that gonococcal attachment-ingestion and killing by polymorphonuclear leukocytes (PMN) are independent of piliation (153). This increased affinity by T_4 gonococci only occurred with PMN and was reduced to previously reported attachment levels of T_4 gonococci, with other eucaryotic cells. Swanson suggested that a protein other than pili is responsible for attachment of T_2 and T_4 gonococci to PMN's (trypsin treatment of both types decreased attachment), and that pili are not responsible for resistance to phagocytosis (155,156).

Most researchers have reported that all colonial types of gonococci are killed once inside PMN's (126,168,169,177,183). It has also been noted that PMN's of lower animals have a superior gonococcidal action (increase in phagocytosis) than human PMN's and thus might be a factor in animal resistance to gonococcal infection (166,169).

In tissue cell cultures other than PMN's, it appeared that gonococci were ingested by phagocytic action and hence were protected from the action of antibiotics and the host's immune system (87,156,

161,174). In the perfusion system studies using human fallopian tubes, epithelial cells were found to contain in some instances >100 gonococci and it might be possible that intracellular gonococci were multiplying. The end result was destruction of the epithelial cell and exfoliation (182). In synovial tissue biopsy, gonococci were demonstrated within the synovial lining cells (54). It was found that gonococcal antibody to T_2 cells opsonizes both T_1 and T_2 cells for phagocytosis by PMN's (129), and that there is a difference in resistance of gonococci in urethral exudate and subcultured gonococci to the complement-mediated bactericidal action of human sera. Early reports indicated that gonococci from urethral exudate were more resistant than subcultured gonococci (181), but it was found that T_1 gonococci subcultured many times on medium containing prostate extract were more resistant to complement than a T_1 culture recently isolated from urethral exudate (184). From this information it appears that there is yet another mechanism of virulence, which is altered during selective subculture on usual gonococcal media.

Endotoxin and Virulence

Finally it is possible that the lipopolysaccharide endotoxin of *N. gonorrhoeae* could also possibly have a relationship to virulence. Flynn and McEntegart (50) found that in mice gonococcal endotoxin caused a leukopenia and allowed a fatal staphylococcal infection to occur. These results suggested that endotoxin might have an inhibitory effect on migration of phagocytic cells to the site of infection. It is not known whether the extensive tissue damage in gonococcal infection is due to endotoxin or to acute inflammation due to

lysosomal enzymes of PMN's. Recent evidence (182) suggests that endotoxin might contribute to damage and exfoliation of epithelial cells in the absence of PMN's, due to the fact that no attachment or ingestion of gonococci by ciliary epithelium during infection has been recorded, although these cells also become damaged and exfoliate.

Identification and Cultivation

Neisseria gonorrhoeae is the cause of a number of contagious infections of human columnar and transitional epithelium with a special predilection for mucosal membranes. Urethritis and cervicitis are usually thought of as the primary infections of importance, but now salpingitis (132), vulvovaginitis (128), arthritis (4,67), ophthalmia neonatorum (91), peritonitis (128), endocarditis (70, 82), epididymitis (128), pharyngitis (5,34,187), hepatitis (70), meningitis (70), and dermatitis (1,14) are becoming more frequent manifestations. There are certain other organisms that may also cause some of these diseases and consequently need to be differentiated from the gonococcus. The organisms that can most easily be mistaken for *N. gonorrhoeae* are usually destained staphylococci and streptococci, and members of other genera in the family Neisseriaceae. The staphylococci and streptococci can most easily be differentiated by their typical morphology and biochemical reactions which do not resemble those of the gonococcus. The other genera of the family Neisseriaceae--*Branhamella*, *Moraxella* and *Acinetobacter* (135)--show only subtle differences biochemically and morphologically (see Table 2) compared to *N. gonorrhoeae*. *Acinetobacter calcoaceticus* var. *lwoffi* has been found to cause a gonorrhea-like urethritis (84,150) and

Table 2.* Differential characteristics of the genera of family Neisseriaceae[a]

	I. Neisseria	*II. Branhamella*	*III. Moraxella*	*IV. Acinetobacter*[b]
Cells	coccal	coccal	plump rods in pairs or short chains	rod-shaped in exponential phase but nearly spherical in stationary phase
Division	two planes	two planes	one plane	one plane
Growth in simple defined media	d	-	d	+
Penicillin	originally sensitive	originally sensitive	originally sensitive	originally sensitive
Oxidase	+	+	+	-
Reduction of nitrate	- or d	+	d	-
G+C moles %	47-52	40-45	40-46	39-47

*From *Bergey's Manual of Determinative Bacteriology*, 8th ed., The Williams and Wilkins Company, Baltimore, 1974.

[a]+ = most strains positive (≧90%); - = most strains negative (≧90%); d = some strains negative, some strains positive.

[b]Temporarily associated with Neisseriaceae (Report Subcommittee on Taxonomy of *Moraxella* and Allied Bacteria 1971. Int. J. Syst. Bacteriol., 21:213. Min. 13).

Moraxella sp. and *Branhamella catarrhalis* can cause conjunctivitis (68,157), and therefore it is possible that many of the reported penicillin-resistant gonococcal strains may have been those organisms just referred to, if proper procedures of identification were not carried out.

Once differentiation between the genera has been made, speciation of the *Neisseria* is also necessary. Bergey's *Manual of Determinative Bacteriology* (135) includes six species in the genus *Neisseria*, viz., *N. gonorrhoeae*, *N. meningitidis*, *N. sicca*, *N. subflava*, *N. flavescens* and *N. mucosa*. There are also ten other tentative species mentioned. *Neisseria meningitidis* has been isolated from the vagina and cervix of patients without clinical signs of disease (101) and the other species are part of the normal flora of the nasopharynx. One of the proposed species, *N. lactamicus*, has also been found to grow on media selective for the pathogenic species *N. gonorrhoeae* and *N. meningitidis*, and inhibitory to the other commensal species (42). Speciation depends on "fermentation" of carbohydrates, growth requirements--nonenriched, nonselective vs. enriched, selective nutrient agar--and colonial morphology (see Table 3). In most cases differentiation is probably most critical between *N. gonorrhoeae*, *N. meningitidis* and the proposed *N. lactamicus*. Much has been published on fermentation media (51,81,160,167) describing efforts to overcome the problem of nonfermentation of glucose by about 0.05% of the gonococci (133), and nonspecific and weak reactions that are difficult to interpret (135). The Center for Disease Control (CDC) recommends the use of cystine trypticase agar (BBL) containing a final

Table 3.[@] Characteristics differentiating the species of genus *Neisseria*[a]

	1. N. gonorrhoeae	*2. N. meningitidis*	*3. N. sicca*	*4. N. subflava*	*5. N. flavescens*	*6. N. mucosa*
Capsules	-	v	v	+	-	+
Acid from:						
Glucose	+	+	+	+	-	+
Maltose	-	+	+	+	-	+
Fructose	-	-	+	v	-	+
Sucrose	-	-	+	v	-	+
Starch	-	-	v	v	-	+
Polysaccharide produced from 5% sucrose	0	0	+	d	+	+
Production of H_2S	-	-	+	+	+	+
Reduction of:						
Nitrate	-	-	-	-	-	+
Nitrite	-	d	+	+	+	+
Pigment	-	-	d	+	+	-
Extra CO_2	//	/	*	*	*	*
Growth at 22 C	-	-	d	d	+	+
G+C moles %	49.5-49.6	50.0-51.5	49.0-51.5	48.0-50.5	46.5-50.1	50.5-52.0

[@]From *Bergey's Manual of Determinative Bacteriology*, 8th ed., The Williams and Wilkins Company, Baltimore, 1974.

[a]+ = most strains positive (≧90%); - = most strains negative (≧90%); d = some strains positive, some negative; v = character inconstant and in one strain may sometimes be positive, sometimes negative; 0 = no growth on medium with 5% sucrose; g+C = guanine + cytosine in the deoxyribonucleic acid; / = not tested; // = very important; * = not necessary.

concentration of 20% solution of the sugars for fermentation studies (162). Recently a new rapid method has been developed in which a loopful of a suspension of gonococci in buffered saline is added to 0.1 ml buffered saline-phenol red indicator and 1 drop of 20% carbohydrate solution. The test is read in 1-4 hours (20,85). Fluorescent antibody is useful for identification in diagnostic work (13,37,77,125,127,134,171,185), but antisera must be absorbed with *N. meningitidis* to avoid cross reactions (136).

Clinical diagnosis in males can be made on the basis of symptoms and the presence of intracellular (PMN) Gram negative diplococci in a Gram-stained smear. Definitive diagnosis requires identification by cultural or fluorescent antibody methods. Gram stains are of no value in the diagnosis of the disease in female patients and therefore only the results of cultural and fluorescent antibody procedures are acceptable (162). Specimens should be cultured immediately on specific selective media or dispatched to the laboratory as soon as possible in a transport medium. Transgrow (20,32, 112), a culture bottle containing Thayer Martin or modified Thayer Martin media and CO_2 (111 and see below) has proved to be a good transport medium in many studies (35,72,170).

Primary isolation of *Neisseria* can be achieved using certain commercial media and under specified temperature, pH and CO_2 conditions. Growth of *N. gonorrhoeae* may be obtained within a range of 30-38.5°C with optimum growth of most strains occurring at 35-36°C (135). One researcher obtained high yields of T_2 colonies at 30°C (76). Gonococci are able to grow in a pH range of 5.8-7.4 with

best growth claimed at 7.3 in one report and 6.75 in another (18, 19,74). Above and below the claimed optimum pH, lysis was reported to occur (18,19). Moisture has been found to increase gonococcal growth (47,114,135), but excessive moisture, as produced by syneresis of agar, is undesirable both for primary isolation and especially for subculture, making colonial types undifferentiable (84,163). The gonococcus is rapidly killed by drying, sunlight, ultraviolet light, moist heat at 55°C within 5 minutes, and also by solutions of phenol, bichloride of mercury and silver compounds (142,163).

Increased CO_2 tension has been found to stimulate aerobic growth of *N. gonorrhoeae* (47,64,114). Media are best incubated in a candle jar containing approximately 3.3% CO_2 (47,102,186) or in a jar whose atmosphere is enriched with 8-10% CO_2 (47,133). It has been reported that high CO_2 tension favors the growth and stability of T_1 and T_2 colonies on solid media (76,84). Some gonococcal strains were able to multiply in the presence of air only but at much slower rate than when under increased CO_2 tension (29,63).

One of the first media designed for primary isolation of *N. gonorrhoeae* was the casamino acid-starch agar of Mueller and Hinton (122). Moller and Reyn (117) then devised an agar medium containing protein-free beef heart broth, yeast and liver autolysates, and hemoglobin, but the most effective and easiest medium to prepare was developed by Thayer and Martin (164). The recently modified medium (165) consists of Bacto GC medium base, hemoglobin, yeast supplement B and the antibiotics vancomycin (inhibits Gram positive organisms), colistimethate sodium (inhibits Gram negative organisms) and nystatin

(inhibits yeasts). Further modification included the addition of the antibiotic trimethoprim to supress the growth of spreading *Proteus* and substitution of Isovitelex enrichment (BBL) for yeast supplement B (111). Some gonococcal species have been found to be sensitive to vancomycin; therefore, use of Thayer Martin medium with and without antibiotics is advised for primary isolation (136). Peizer medium modified by Amies and Garabedian (3) and NYC medium of Faur *et al.* (44,45,46) are two other selective media that were developed but are not as widely accepted. These media are essentially the same as Thayer Martin medium except that NYC medium contains yeast dialysate, which seems to satisfy some of the CO_2 requirements of the gonococcus, and amphotericin B instead of nystatin. The selective properties of all these media that incorporate antibiotics have greatly increased the recovery of gonococci from clinical specimens (163).

Much work has been done on defining growth requirements and inhibitors of *N. gonorrhoeae*. Glutamine (98) and casein hydrolysate (62) were some of the first nutrients found to enhance gonococcal growth. Lankford (97), in 1950, found that 23.3% of species tested required glutamine, 0.8% required carboxylase and 0.5% required unidentified nutrients. Tryptophan and biotin were found to reduce the lag period in gonococcal growth curves, while biotin and oxaloacetic acid were found to act as a source of CO_2 for the gonococcus (64). Kellogg, in 1963, discovered that ferric nitrate or ferric ions increased colony color and growth rate (83,84). More definite requirements were elicited when Hunter (74), in 1970, added cystine,

proline, arginine, leucine, isoleucine, valine, threonine and glutamic acid to her chemically defined media (see below) because of their necessary and stimulatory effect on growth. Cystine appears to be one of the essential nutrients, and it was noted by Lankford (97), by Hunter (74), and also by Catlin (27) that absolutely no growth was obtained without it with some strains.

In the early 1940's Gould noted that several amino acids--glycine, tryptophan, tyrosine, cystine and adenine--exerted a toxic effect on *N. gonorrhoeae* (62). Tween 80 was found by Diana (39) to lyse gonococci in concentrations of 1:2,500 and 1:5,000 and Dubos Oleic acid agar was completely inhibitory, although recently Catlin incorporated 0.002% extracted Tween 80 in her NEDA (29). One source reported that autolysis of gonococcal cells was due to depletion of energy sources, rather than to an addition of inhibitors (118). Many researchers have reported the inhibitory effect of ordinary agar (11,62,74,100,103) and recommended the use of purified agar or the addition of starch as an absorbant (62,100,103). Ley and Mueller (103) isolated a fatty acid-like inhibitor from agar and recommended the addition of starch to media containing agar because of the ability of its linear component to adsorb free fatty acids. Recently it was also found that gonococci produce self-inhibitory phospholipids--monoacyl phosphatidylethanolamine, and long chain free fatty acids (176). This inhibitory action was completely blocked by the addition to the media of 2% (wt/vol) bovine serum albumin and partially blocked by the addition of 1% soluble starch. Serum albumin has been reported to have a higher affinity for fatty acids than starch (36).

Also of importance were the inhibitory effects of micromolar amounts of cupric salts and the copper coil intrauterine device (48,33). Copper supposedly reacts with membrane enzymes and alters membrane permeability. Progesterone also has an inhibitory effect (120).

Several chemically defined media have been developed for the genetic and biochemical study of the gonococcus. One of the first was NCDM medium containing Medium 199, $NaCO_2$, carboxylase, L-glutamine, dextrose and ferric chloride (88,90). This medium and another devised by Hunter *et al.* (74) were not optimal for all strains because of the great strain variability in response to amino acids. A maximum medium, NEDA, was developed by Catlin (27,29) and afforded luxuriant growth of all strains tested and enabled her to separate her strains into 22 different auxotypes with regard to L-proline, L-arginine, L-ornithine, L-methionine, hypoxanthine, uracil, thiamine and thiamine pyrophosphate requirements. La Scolea and Young (100), using Gonococcal Genetic Medium, a minimum nutritional medium, were able to classify strains into 8 major and minor groups using 5 amino acids--cysteine and cystine, arginine, proline, isoleucine and serine. They were able to support Catlin and associates in finding two major phenotypic classes--cysteine and cystine requirers and proline requirers (27,100).

Currently the most efficient method of growing *N. gonorrhoeae* and of maintaining colonial type is the biphasic system of Gerhardt and Hedén (57) consisting of a flask containing 100 ml of agar and 25 ml of a broth overlay. Several researchers have obtained optimal

yields of cells and greater stability of T_1 and T_2 colonial types using this system (74,76,99).

Neisseria gonorrhoeae has also been grown in tissue culture using HeLa, RE_2, KB, rat embryo, mouse 313, vero, LLC-MK, and WI-38 cells (10,55,89,94,172) and also in organ cultures (28,182). The gonococci attach to and are ingested by the tissue culture cells and cause detachment of the cells from the glass (55). In one instance (55) viability was maintained in tissue culture for 88 days after first inoculation.

Hill has a very extensive review on the research animals in which propagation of gonococci was attempted (69). Until recently gonococcal growth was maintained only in the anterior chamber of the rabbit's eye (116). Recently, researchers have obtained growth of gonococci in hollow polyethylene "practice golf balls" (6), in small lengths of polyethylene tubes (143), and in coils of wire and vinyl (7,8,52) implanted subcutaneously in rabbits, guinea pigs, hamsters, mice and rats. After 2 days a mild foreign body reaction occurred and in approximately 2 weeks granulation tissue formed a walled-off chamber filled with serous transudate in which T_1, T_2, and T_4 gonococcal colony types grew. Infection in these coil and golf ball chambers has been maintained for up to 9 months, with the appearance of serum antibody in 6-30 days. When infection was allowed to terminate naturally in the chambers formed with polyethylene tubes and subsequently rechallenged with 10^8 colony forming units/ml *N. gonorrhoeae*, no growth occurred (143). In chick embryos, growth of *N. gonorrhoeae* has been demonstrated in the chorioallantoic

cavity (26,58) and intravenously (24). Studies with these embryo models support the correlation of virulence and T_1 and T_2 colonial types. Gibbs *et al.* (58), using the chicken embryo for quantitative *in vitro* studies of phagocytosis, were able to obtain log phase growth, stability of colonial types and absence of clumping, after cultivating the gonococcus in the allantoic cavity of 10-day chick embryos. The most promising and applicable model has been the chimpanzee. Infection has been experimentally established in the chimp using pooled urethral exudate (105), subcultured gonococci (23) and also has been transmitted between chimps by coitus (23,105). Immunological studies showed CF antibodies appeared earliest and tests gave positive reactions when infection was not detectable by culture (22). Chimpanzees were also systemically immunized with formalinized whole gonococcal cells (vaccine) and demonstrated an increased resistance to rechallenge with the same strain, although susceptibility to infection was still demonstrable when a different strain of *N. gonorrhoeae* was utilized (9).

Propagation of *Neisseria gonorrhoeae* in Dialysis Culture Systems

Dialysis culture of bacteria began in 1896 when Metchnikoff and his associates implanted collodion sacs containing cultures of *Vibrio cholera* into peritoneal cavities of various animals to demonstrate the presence of a diffusible toxin (115). Since then there has been much experientation on *in vivo* and *in vitro* dialysis culture of bacteria and comprehensive historical reviews were compiled by Schultz and Gerhardt (144), Humphrey (73), and Quarles (130). Over 50 different kinds of algae, bacteria, fungi, protozoa and tissue

cells have been studied in *in vitro* dialysis culture (144) and 7 genera of bacteria have been studied *in vivo* (130).

The main advantages of propagating bacteria in dialysis culture are: the prolonged period of active reproduction by the organism, resulting in a higher density of viable cells; the stabilization of the maximum stationary phase of the organism's growth cycle; the removal or dilution of diffusible inhibitory growth products; the fact that cultures can be grown free from macromolecules of growth media; and that interactions between separate populations of microbial and/or tissue cells can be studied (144).

The three components usually thought to compose a model dialysis system include the reservoir, the membrane-interface diffusion barrier, and the culture chamber. Schultz and Gerhardt (144) have commented that there are many different ways in which these components can be designed to define a dialysis culture system. A number of modifications employ interface dialysis using the interface between two different phases as a diffusion barrier. Examples of these would include agar-agar, gas-liquid, gas-solid interfaces, etc. The biphasic system of Gerhardt and Hedén (57) employed in propagation of *N. gonorrhoeae* is an example of a solid-liquid dialysis culture system. Compared with other methods of cell propagation, this system has produced the highest yield of cells (99).

Membrane dialysis incorporates sheets, tubes or sacks fabricated from collodion, parchment, cellophane or a variety of plastics as the diffusion barrier. Variations of membrane dialysis used in *in vivo* propagation and virulence studies with *N. gonorrhoeae*

included the golf ball and coil technique of Arko in which a natural diffusion barrier was formed by fibrous connective tissue (6,7,8, 52,143), and the use of the chorioallantoic membrane (CAM) of a chicken embryo by Buchanan, itself a natural diffusion membrane (24). Disadvantages to the method used by Arko include the nonspecific immune response that may be elicited by the artificial chamber exclusive of bacteria and the fact that the chambers are "walled off" making diffusion minimal. The "golf ball", coil and CAM models are difficult to sample without sacrificing the host.

MATERIALS AND METHODS

General Media and Reagents

Neisseria gonorrhoeae was selectively subcultured daily on GC Agar Base (BBL) plus 1% Isovitelex Enrichment (BBL). The ingredients of the GC Agar Base were 1.5% Proteose Peptone No. 3, 0.4% K_2HPO_4, 0.1% KH_2PO_4, 0.5% NaCl, 0.1% corn starch, and 1.0% agar; and of the Isovitelex Enrichment: 0.001% vitamin B_{12}, 1.0% L-glutamine, 0.1% adenine, 0.003% guanine hydrochloride, 0.0013% p-aminobenzoic acid, 0.11% L-cystine, 10% dextrose, 0.025% diphosphopyridine nucleotide oxidase (Coenzyme 1), 0.01% cocarboxylase, 0.002% ferric nitrate, 0.0003% thiamine hydrochloride, and 2.59% cysteine hydrochloride. GC Agar Base plus 1% Isovitelex Enrichment (GCA) was incubated for 24 to 48 h at 37°C before use, to eliminate excess moisture caused by syneresis of the agar. Plates were only used for a week to 10 days. After this period some degradation of the media inhibited gonococcal growth. GC Broth (GCB) was prepared with the same ingredients contained in GCA with the exception of agar and corn starch. The gonococci used in the study were tested for oxidase activity using Taxo Discs (BBL)--Differentiation Discs for *Neisseria* and *Pseudomonas*. The production of acid from glucose only was confirmed using Cysteine Trypticase Agar (BBL) plus 20% solutions of glucose, maltose, sucrose, or lactose.

Media and Reagents Used in Experiments with the Marbrook Chamber

GCB was the basic culture medium used in the Marbrook Chamber. Different materials were added to the GCB in numerous experiments to compare their effects on the gonococcus. The various additives were: 0.1% Daxad 23 (Dewey and Almay Chemical Co.), 0.25%, 0.1%, 0.01%, and 0.002% extracted Tween 80 (Nutritional Biochemical Corp.), 0.1% cornstarch (Sigma), 1% soluble starch (Difco), 2% lyophilized bovine serum albumin (Sigma) and, in one experiment, the concentration of NaCl in GCB was decreased to 0.3% for comparison to the normal GCB NaCl concentration (0.5%).

To remove free fatty acids Tween 80 was extracted using the procedure of Dole (41). Fifty milliliters of extraction mixture (40 parts isopropyl alcohol, 10 parts heptane, 1 part 1N sulfuric acid--all solvents redistilled) was added to 10 ml of Tween 80. The mixture was vigorously shaken and then, after not less than 10 minutes, 20 ml of heptane and 30 ml of H_2O were added. The mixture was shaken again and the top phase containing the extracted lipids was discarded. The resulting extracted mixture was steamed 10 minutes to volatilize the heptane and isopropyl alcohol. Tween 80 in specified amounts was then added to GCB before autoclaving.

Daxad 23 was also added to GCB before autoclaving. Lyophilized bovine serum albumin (BSA) was rehydrated with double distilled water and filter sterilized using a Nalgene Filter Unit before adding to sterilized GCB. The cysteine phosphate buffered saline (CPBS) employed in the final experiment with the Marbrook Chamber contained 0.1% L-cysteine hydrochloride (Sigma), 0.4% K_2HPO_4, 0.1% KH_2PO_4 and 0.85% NaCl (pH 6.9).

Media and Reagents Used in Experiments with the Prosthetic Hemodialysis Culture Unit

White and red blood cell counts (WBC, RBC) of the goat's blood were performed as per Wintrobe (189). Blood was diluted 1:20 with 0.1% HCl for WBC counts, and 1:200 with 0.85% NaCl for RBC counts. Cells were counted on a Neubauer counting chamber.

Blood glucose was determined enzymatically using Glucostat (Worthington Biochemical Corp.), a test containing a colorimetric reagent which specifically breaks down B-D-glucose to H_2O_2 and gluconic acid; H_2O_2 in the presence of peroxidase oxidizes the chromogen present in the Glucostat reagent. The quantitative result was read using a Coleman III, Vis and UV Spectrophotometer (Hitachi Perkin-Elmer).

Samples taken at selected intervals from the Prosthetic Hemodialysis Culture Unit were diluted with GCB or Trypticase Soy Broth (BBL) and then surface plated for viable counts. Goat serum used in the dialysate culture chamber of the Prosthetic Hemodialysis Culture Chamber and in heparin viability tests was obtained from goat's blood clotted in sterile tubes. The serum was sterilized by passage through a Swiminex Millipore Filter (0.22 μ pore diameter).

The phosphate buffered saline (PBS) employed in the dialysate culture chamber consisted of 0.85% NaCl, 0.174% K_2HPO_4, and 0.272% KH_2PO_4 (pH 6.7). The solution utilized in the final experiment with the Prosthetic Hemodialysis Culture Unit was similar in composition to solutions used in human dialysate (130). It contained 0.0101% $MgCl_2 \cdot 6H_2O$, 0.0184% $CaCl_2 \cdot 2H_2O$, 0.05% glucose, 0.517% $NaC_2H_3O_2$, 0.566% NaCl, and 0.0075% KCl.

Sodium heparin (Upjohn Co.) was used to prevent clotting in the goats. Six thousand USP units were injected subcutaneously 2 times per day for a total dosage of 12,000 USP units in 24 h. Sodium heparin was added to GCA in the experiment to determine heparin sensitivity of *N. gonorrhoeae*.

Media Used in Experiments with the Biphasic and Liquid Flask Culture Systems

The Biphasic Culture System consisted of a 250 ml Erlenmeyer flask containing 100 ml GCA, supplemented with 1% Noble Agar (Difco)--total agar concentration of 2%--and overlaid with either 25 ml of GCB or 25 ml of CPBS prepared as previously described. The Liquid Flask Culture consisted of a 250 ml Erlenmeyer flask containing 125 ml GCB plus 1% soluble starch or 125 ml CPBS.

Culture

Neisseria gonorrhoeae strain 2686, colony type T_2, was obtained from W. Jerry Brown of the Center for Disease Control, Atlanta, Georgia. Colony type T_2 was selectively subcultured every 18 to 24 h on GCA. One T_2 colony was selected for subculture using a stereoscopic microscope with diffused, angled light, transmitted from below, up through the medium. The gonococci were incubated in a candle jar at 37°C with a moistened paper towel placed inside the candle jar to supply low humidity. Cultures were preserved by lyophilization or frozen at -20°C in Trypticase Soy Broth plus 20% glycerol (99).

The inoculum used in the experiments was prepared as follows: one T_2 gonococcal colony was removed from subcultured plates using

a sterile cotton swab and suspended in 1 ml GCB. The suspension was then vortexed for 10 seconds and 0.01 ml was surface plated on GCA. After 18 to 24 h the T_2 colonies were harvested with a sterile cotton swab and suspended in a specific amount of GCB according to each experiment.

The percentage of T_2 colony type was calculated by determining the mean number of T_2 colonies per 100 colony forming units (CFU) from each of three dilution plates.

Growth Experiments with the Marbrook Chamber

The Marbrook Chamber as devised by Marbrook (108) was modified in this study by the substitution of a ring of rubber tubing for surgical silk to hold the dialysis membrane in place (Figures 1 and 2). The inner 5 inch length of glass tubing, covered at one end by the dialysis membrane, formed the culture reservoir, and was suspended by a plastic foam plug inside a 50 ml glass test tube which formed the medium reservoir. The inner tube was sealed with a plastic foam plug also. A Dupont 315 cellophane membrane, pore diameter approximately 5 nm, was used. The integrity of the membrane was checked by placing 20 to 25 ml of deionized water in the medium reservoir and observing for leakage of the water into the culture reservoir over a period of 12 h. After sterilization, 15 ml of GCB plus either corn starch, soluble starch or bovine serum albumin was added to the medium reservoir and 1.4 ml of GCB or CPBS was added to the culture reservoir.

An inoculum plate was prepared as previously described and, after 20 h incubation, the T_2 colonies were harvested with a sterile

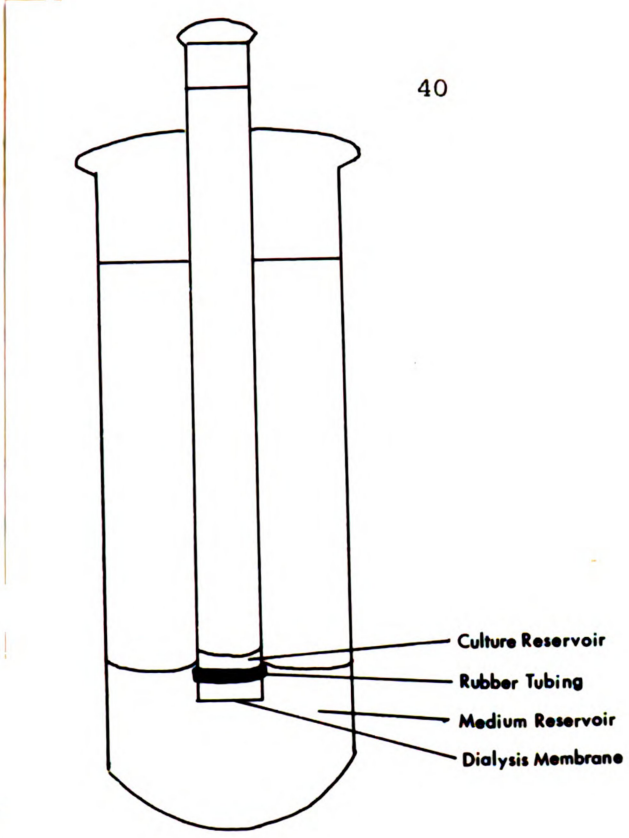

Figure 1. Diagram of Modified Marbrook Chamber.

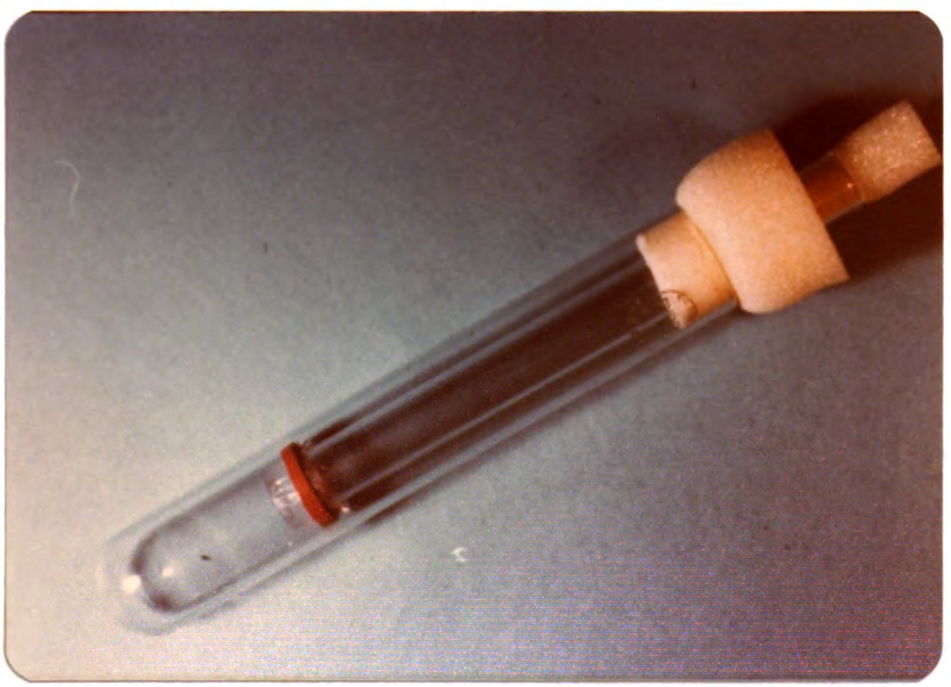

Figure 2. Modified Marbrook Chamber.

cotton swab and suspended in 1 ml (early experiments) or in 5 ml GCB, and either vortexed or sonicated for 20 watts/second for 2 seconds, according to the experiment. One tenth milliliter of the suspension was then added to the culture reservoir of the Marbrook Chamber. Optimum sonication (Biosonik III, Bronwill Scientific) frequency and duration were determined by sonication of 1.5 ml samples for 35, 20 and 10 watts/second for 2 and 5 seconds.

The inoculated Marbrook Chamber was incubated in a 36°C hooded gyrotory water bath shaker (New Brunswick Scientific). Three and three-tenths percent CO_2 was supplied by the use of a candle and the rotation speed was maintained at 240 rpm. No difference in CFU/ml was obtained when the chamber was preincubated for 12 h in 8-10% CO_2 compared with the 3.3% CO_2 supplied by the candle.

One tenth milliliter samples were taken periodically and diluted in GCB with the final 2 dilutions, surface plated on 3 GCA plates per dilution. All dilution plates were incubated in candle jars at 36°C for 18 to 24 h, after which colony forming units were counted and the total number per dilution plate recorded.

Growth Experiments with the Biphasic Culture System

The Biphasic Culture System (BCS) of Gerhardt and Hedén (51) was revised for this experiment by using 100 ml of GCA plus 1% Noble Agar overlaid with 25 ml GCB in a 250 ml Erlenmeyer flask. The culture was incubated at 36°C in a hooded gyrotory water bath shaker at 150 rpm. A candle again supplied 3.3% CO_2 and the inoculum was prepared as previously mentioned with the exception that 1.0 ml was used as the inoculum. Samples again were taken periodically and

diluted as mentioned above. Preincubation of the BCS in 8-10% CO_2 gave no increase in CFU/ml when compared to the 3.3% CO_2 supplied by the candle.

Growth Experiments with the Liquid Flask Culture

The Liquid Flask Culture system consisted of 100 ml of GCB plus 1% soluble starch or 100 ml of CPBS in a 250 ml Erlenmeyer flask. Incubation, inoculation and sampling procedures were the same as the procedure for the BCS.

Growth Experiments with the Prosthetic Hemodialysis Culture Unit

The Prosthetic Hemodialysis Unit (PHCU) as developed by Quarles (130) briefly consists of four main pieces: a blood chamber, consisting of a plastic block fitted with a silicone rubber tubing having a slotted opening; a Dupont 315 membrane with a pore size of approximately 50 Å; a stainless steel support plate; and the dialysate culture chamber which consists of a hollow plastic block with a volume capacity of 3.3 ml (Figures 3, 4 and 5). The dialysate culture chamber was sampled using a needle inserted into a small hole plugged with a vaccine-bottle stopper. The unit was sterilized with ethylene oxide (Bard Sterilizer System 2279, D. R. Bard, Inc.) for 6 to 18 h and then aired for a minimum of 24 h.

Two short-haired goats of mixed breed were used as experimental animals. The first goat succumbed after the first experiment. Surgery was performed as per Quarles (130,131) and the PHCU was attached to the external arterial-venous shunt by means of Teflon connectors. During the course of the experiments the goat was maintained unrestrained in a small stall.

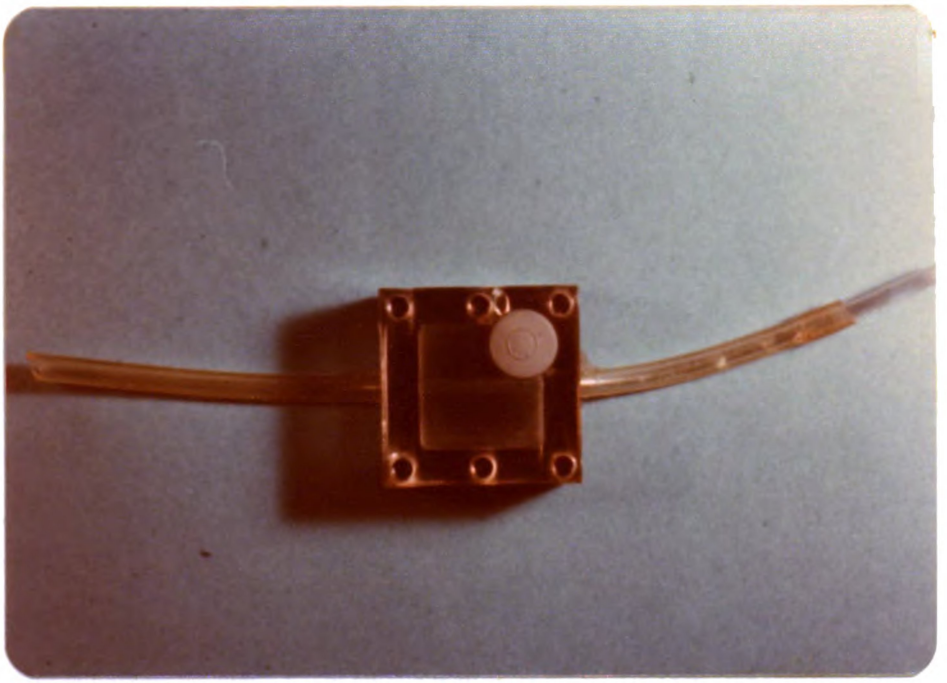

Figure 3. Prosthetic Hemodialysis Culture Chamber: front view.

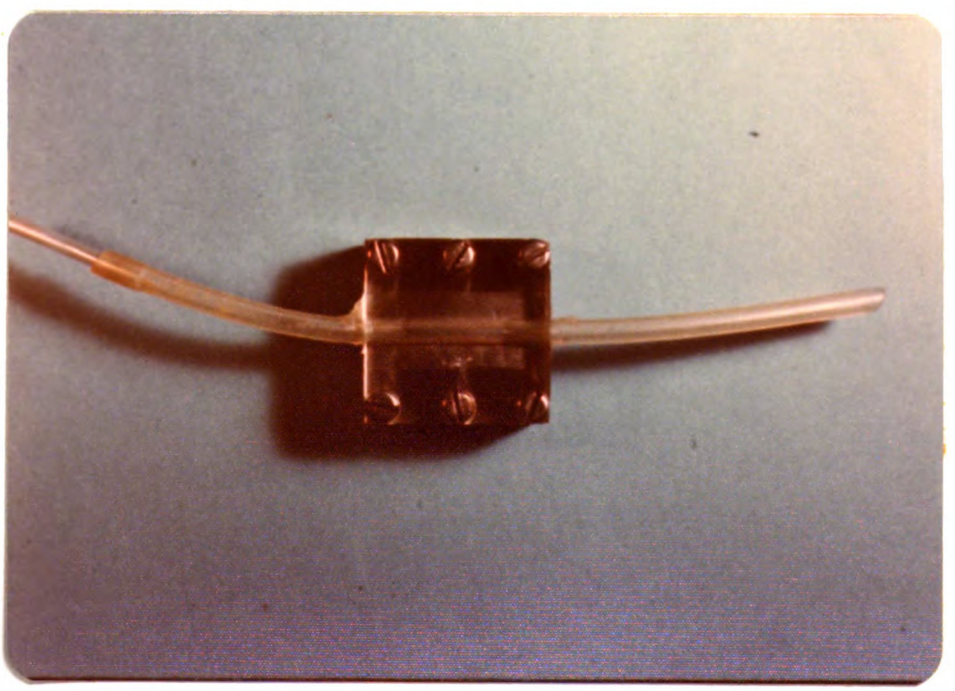

Figure 4. Prosthetic Hemodialysis Culture Chamber: rear view.

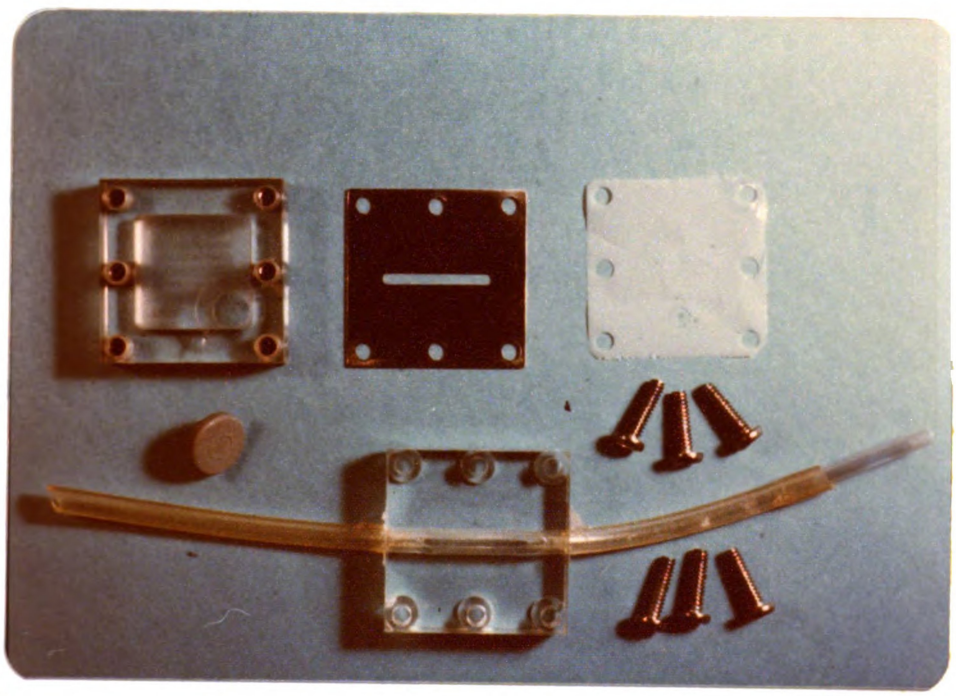

Figure 5. Disassembled Prosthetic Hemodialysis Culture Chamber.

The inoculum plate was prepared as for the Marbrook Chamber and colonies were harvested in 18 to 24 h and suspended in various culture solutions employed in the culture chamber: 0.85% NaCl, goat serum, 25% goat serum in PBS, and human dialysate solution. The inoculum (0.3 ml) was added to 3 ml of culture solution in the dialysis culture chamber. When 0.85% NaCl and human dialysate solution were used as culture solutions the chamber was attached to the goat and allowed to equilibrate for 12 and 4 h, respectively, prior to introducing the inoculum.

One tenth milliliter samples from the dialysate culture chamber were obtained periodically and diluted in either GCB or Trypticase Soy Broth according to the experiment. The two final dilutions of each sample were surface plated (3 plates per dilution) on GCA for viable cell count determination as per experiments using the Marbrook Chamber. In early experiments, rectal temperatures were recorded and samples of blood for glucose, WBC and RBC counts were taken at each sampling interval; but in later experiments monitoring of these functions was not maintained due to lack of significant changes in early experiments.

To test the viability of *N. gonorrhoeae* in heparinized and unheparinized goat serum, an inoculum plate was again prepared and colonies harvested after 20 h and suspended in 1 ml PBS. A 1:10 dilution was made and 0.1 ml was added to 9.9 ml sterile goat serum. Samples were taken at 0 and 2 h in the experiment with heparinized goat serum, and 0, 2 and 24 h in experiments with unheparinized goat serum.

The agar plate dilution method was used to determine the sensitivity of *N. gonorrhoeae* to heparin. Sodium heparin was added to 25 ml sterilized GCA so that the agar plates contained final concentration of 125 U, 250 U and 500 U/ml. Colonies were harvested from an inoculum plate after 20 h incubation and suspended in 1 ml of PBS. A 10^{-7} dilution was surface plated on 12 plates--3 plates for each heparin concentration and 3 plates of GCA without heparin for a control. Colonies were counted and observed under the stereoscopic microscope at 24 and 48 h.

RESULTS

Marbrook Chamber

Preliminary Experiment: Determination of Generation Time and Percent T_2 Colony Morphology. Since *Neisseria gonorrhoeae* was reported to have grown well in the Biphasic Culture System it seemed plausible that growth could be obtained in the Marbrook Chamber. In a preliminary experiment to determine generation time and percent maintenance of T_2 colony forming units, GCB plus 0.1% corn starch was placed in the medium reservoir and GCB inoculated with T_2 gonococcal cells in the culture reservoir. The initial inoculum was 2.05×10^9 CFU/ml and after 10 h the number of CFU/ml was 4.62×10^{11}, after which a decline in CFU/ml was noted. T_2 colony type was maintained above 92% during the first 12 h (Figure 6). After 12 h, the percent T_2 colony type declined to levels well below 90%; therefore only the first 12 h of growth were recorded in all experiments. Generation time for the preliminary experiment was approximately 51 minutes.

Experiment 1: Addition of Daxad 23 to Prevent Agglutination of Gonococci. A problem noted in the preliminary experiment was the agglutination of the T_2 gonococcal cells during growth. Due to this visible clumping the next few experiments were designed to employ

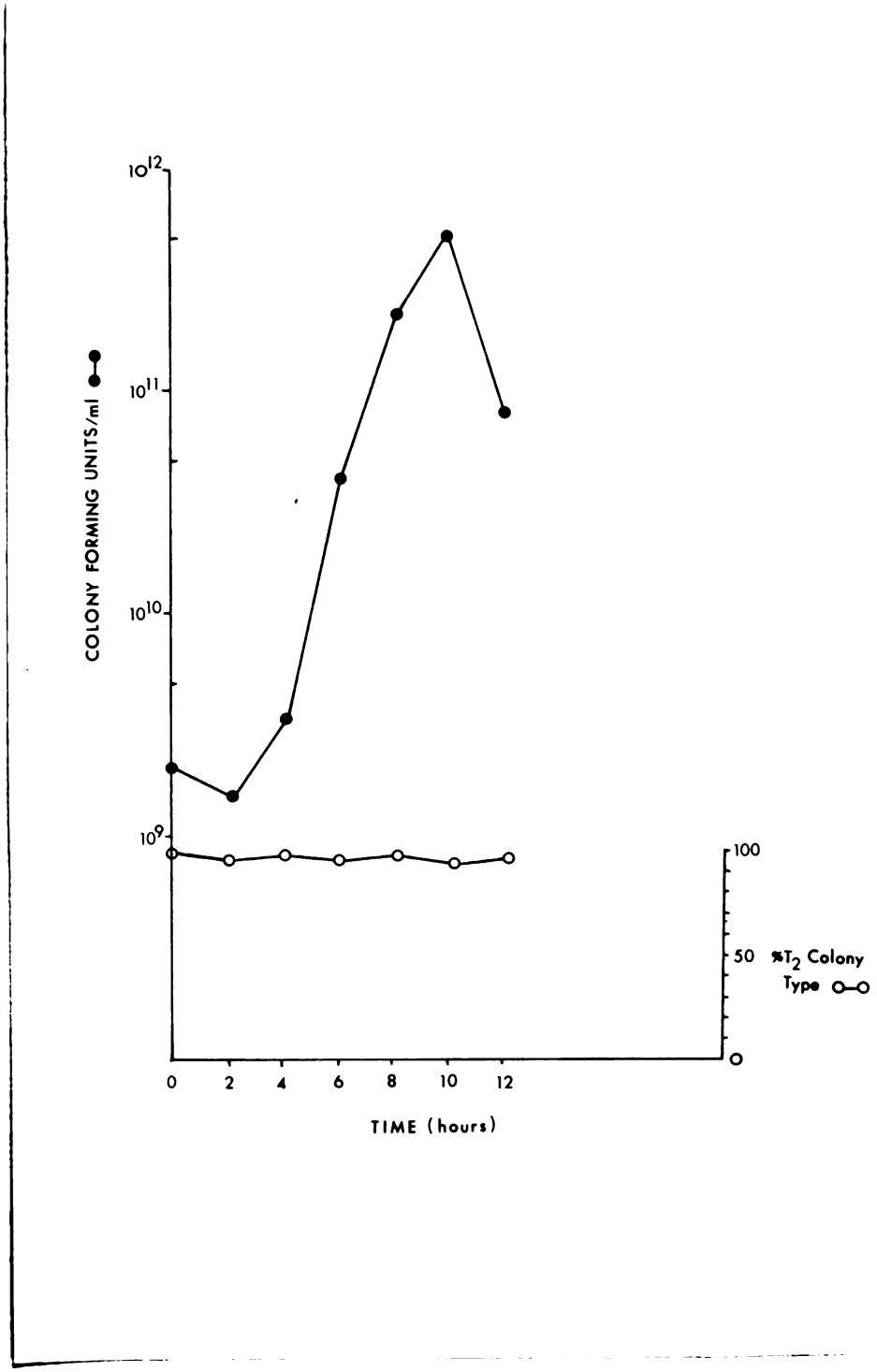

Figure 6. Preliminary dialysis experiment with the Marbrook Chamber to determine generation time and percent T_2 colony morphology.

various materials and techniques which might disperse the agglutinated cells and therefore lend greater accuracy to the surface plate method for viable counts. In the first experiment 0.1% Daxad 23 was employed, but clumping of the gonococci was still visibly evident during growth and after the CFU/ml were counted the addition of 0.1% Daxad 23 seemed to have an inhibitory effect on gonococcal growth, as shown in Figure 7.

Experiments 2 and 3: Addition of Tween 80 and Sonication of Samples to Disperse Agglutinated Gonococci. In the next two experiments, extracted Tween 80 was utilized to see if it would have any effect on dispersing the agglutinated gonococcal cells. In a preliminary experiment testing various concentrations of extracted Tween 80 (0.25%, 0.1%, 0.01%), no growth was obtained with any of the different concentrations after 12 h, and colonies at 0 h were extremely small in size compared to the control Marbrook Chamber making colony type impossible to determine (Table 4). It is interesting that the

Table 4. Effect of 0.25%, 0.1%, and 0.01% extracted Tween 80 on CFU/ml

Hours of culture	CFU/ml MC+0.25%	CFU/ml MC+0.1%	CFU/ml MC+0.01%	CFU/ml MC
0	4.3×10^5	8×10^5	9×10^5	1.1×10^8
12	no growth	no growth	no growth	2.3×10^{11}

MC = Marbrook Chamber.

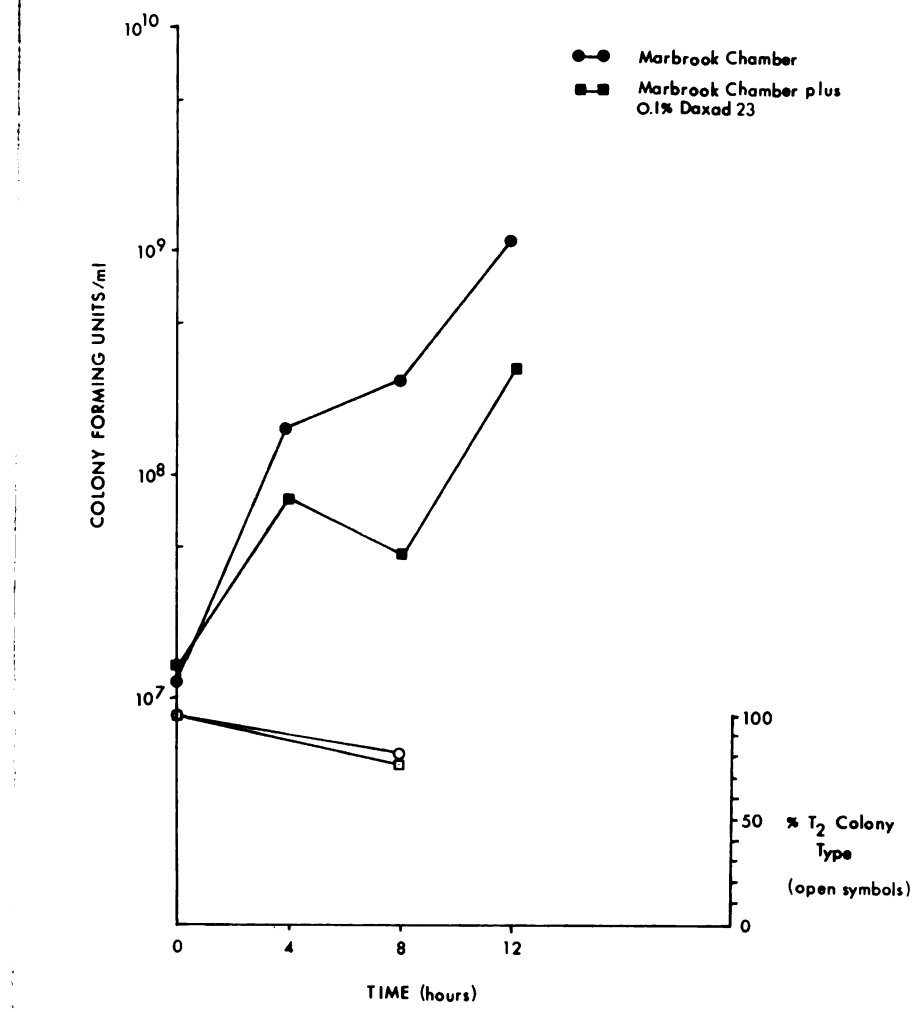

Figure 7. Effect of 0.1% Daxad 23 on CFU/ml and percent T_2 colony morphology.

original inoculum was the same for all the Marbrook Chambers, but was already decreased in Tween 80 samples at 0 h. Catlin (29) employed 0.002% Tween 80 in her chemically defined medium and this concentration was tested in an experiment which also compared sonicating the samples taken from the Marbrook Chamber. Sonication was performed on the initial inoculum before the chamber was inoculated and then on samples after they were taken from the Marbrook Chamber. The chamber itself was not sonicated due to the possibility of disturbing the integrity of the membrane. The frequency and length of sonication were determined in a separate test in which equally divided portions of a 10^{-6} and a 10^{-7} dilution of gonococcal cells were sonicated at 35, 20 and 10 watts/second for 2 and 5 seconds. The results are shown in Figure 8. Samples sonicated at 20 and 35 watts/second for 5 seconds, and 35 watts/second for 2 seconds produced less CFU/ml than a nonsonicated control sample, and it was evident that decreased viability of gonococcal cells was incurred at these frequencies and time durations. Therefore it was decided to use 20 watts/second for 2 seconds as the rate of sonication to be used in this study.

The results of the experiment comparing sonicated and nonsonicated samples from the Marbrook Chamber, and 0.02% Tween 80, demonstrated that 0.002% Tween 80 really had no effect on dispersing agglutinated cells due to no increase in CFU/ml. After sonication of the sample there was an initial increase in CFU/ml at 0 h due to the fact that sonication did disperse agglutinated T_2 gonococcal cells in the inoculum that was the same for all three Marbrook

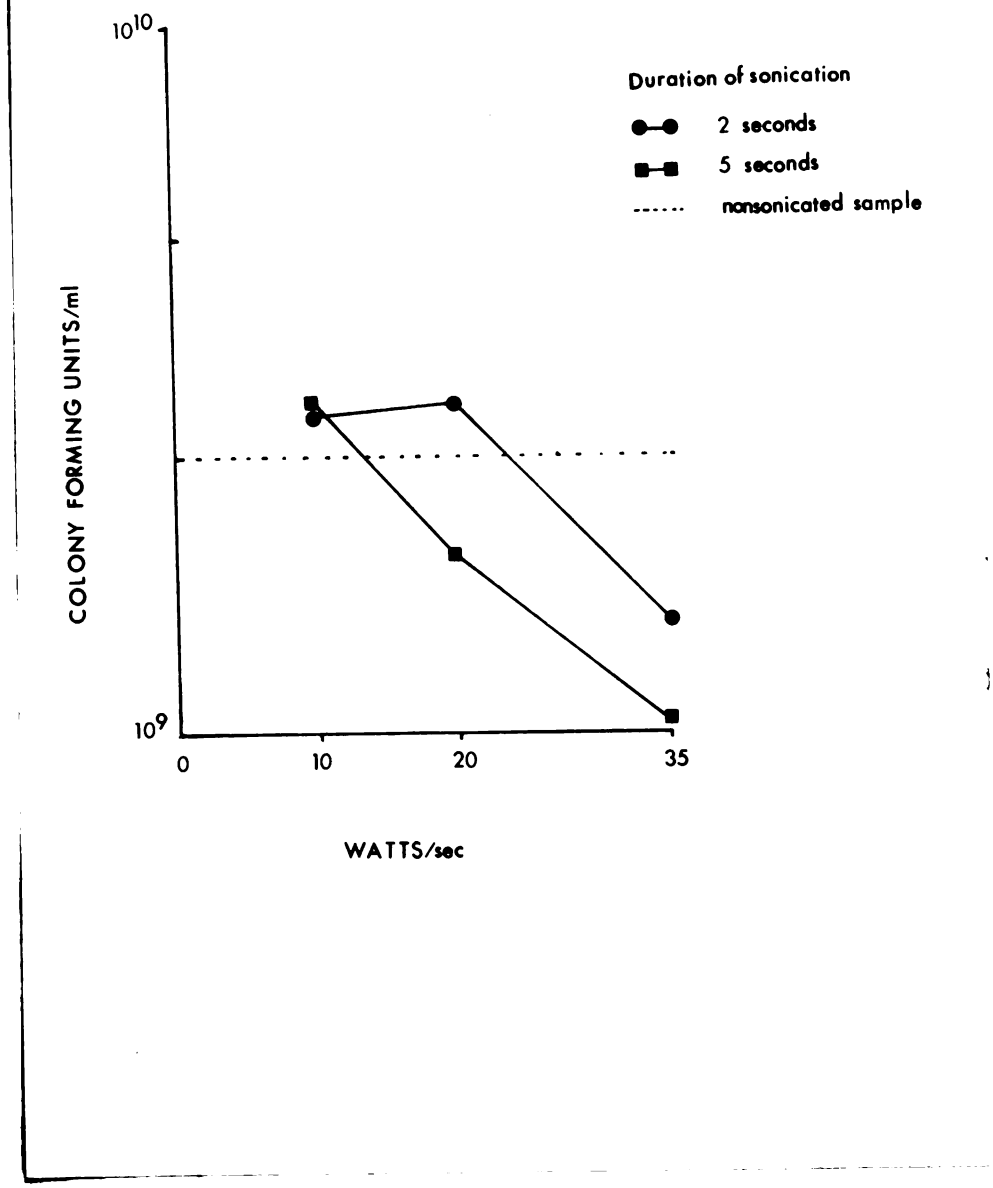

Figure 8. Effect of different sonication frequencies and the duration of sonication on CFU/ml.

Chambers, but after the Marbrook Chamber was inoculated with the sonicated cells, they reclumped and therefore the increase in CFU/ml in sonicated samples after 0 h was not as apparent (Figure 9).

Experiment 4: Decrease in NaCl Concentration to Prevent Agglutination. A final attempt was made to disperse agglutinated cells by decreasing the NaCl concentration of GCB. As shown by the results in Figure 10, there was no significant increase in CFU/ml and percent T_2 colony morphology remained at high levels.

Experiment 5: Comparison of the Effect of Different Adsorbants on CFU/ml and Percent T_2 Colony Morphology. In previous experiments 0.1% corn starch had been used as the adsorbant added to the GCB in the medium reservoir. Because corn starch had tended to precipitate and settle on the bottom of the large tube during experiments, 1% soluble starch and 2% bovine serum albumin were compared with 0.1% corn starch as to their effect on number of CFU/ml. A Marbrook Chamber containing no starch was added as a control. The results (Figure 11) indicate that 2% bovine serum albumin had an inhibitory effect on CFU/ml greater than the inhibitory effect of no adsorbant. Soluble starch and corn starch were of equal effect on CFU/ml and therefore soluble starch was employed in the remaining experiments.

Experiment 6: Comparison of Growth of *Neisseria gonorrhoeae* in the Marbrook Chamber, BCS and Liquid Flask Culture. As mentioned previously, the BCS is an example of a solid-liquid dialysis system, and thus in the final two experiments it was compared with the Marbrook Chamber and Liquid Flask Culture. In the first experiment,

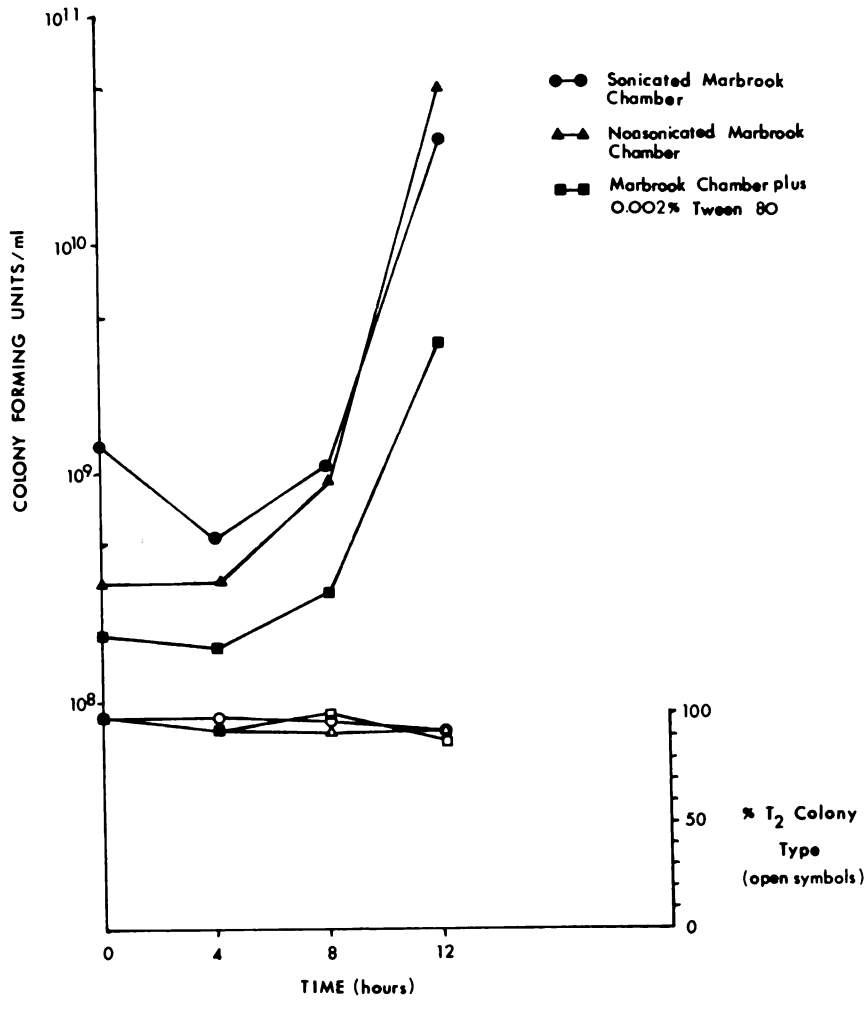

Figure 9. Comparison of effect of 0.002% Tween 80 and sonication of samples on CFU/ml and percent T_2 colony morphology.

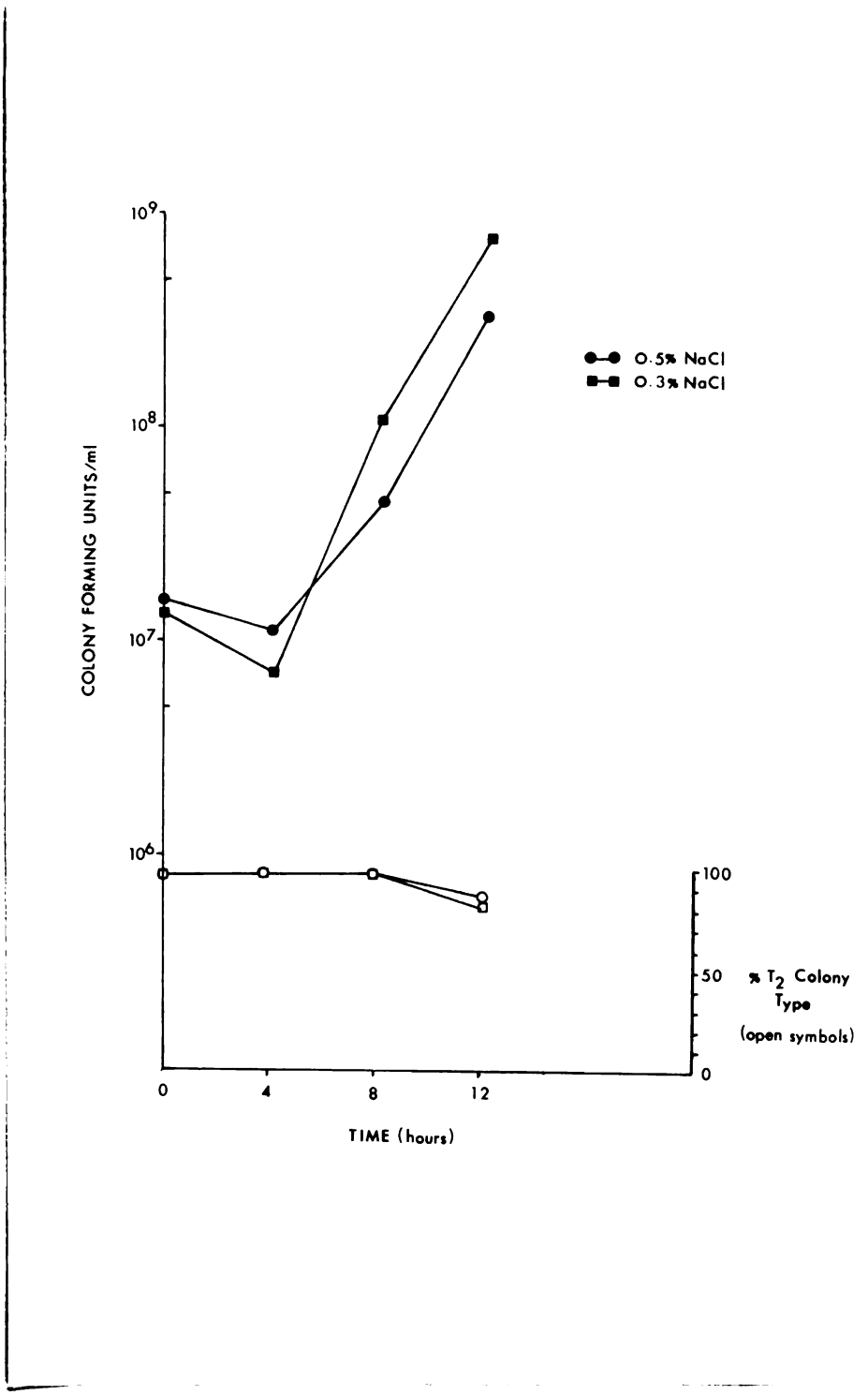

Figure 10. Comparison of effect of 0.3% NaCl and 0.5% NaCl in GCB, on CFU/ml and percent T_2 colony morphology.

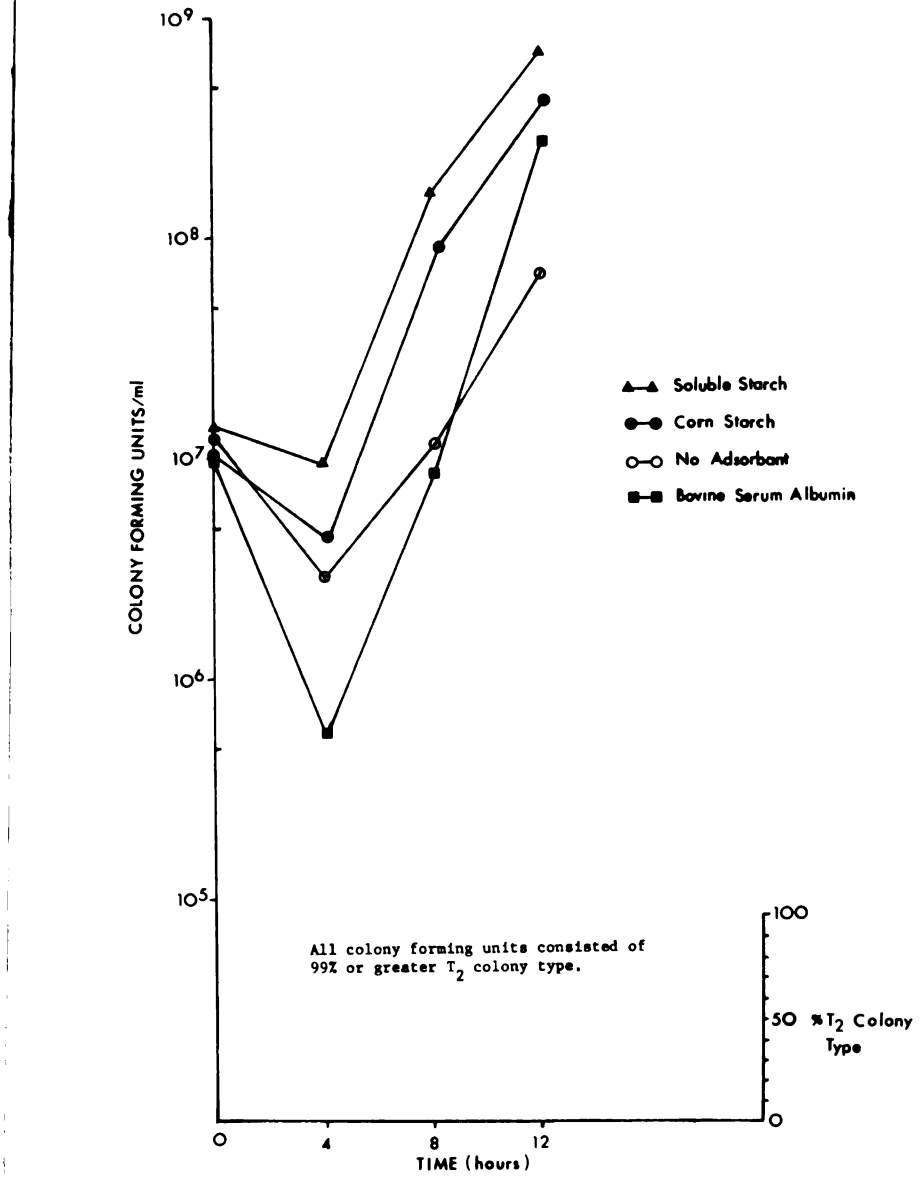

Figure 11. Comparison of effect of 0.1% corn starch, 1% soluble starch and 2% bovine serum albumin on CFU/ml and percent T_2 colony morphology.

the Marbrook Chamber with 1.5 ml GCB in the culture reservoir and 15 ml GCB plus 1% soluble starch in the medium reservoir was compared to the BCS containing 100 ml GCA plus 1% Noble Agar and 25 ml GCB, and 125 ml GCB plus 1% soluble starch in the Liquid Flask Culture. Results depicted in Figure 12 indicate that the Marbrook Chamber did not yield as high a number of CFU/ml as did the BCS or the Liquid Flask Culture.

Experiment 7: Growth of *Neisseria gonorrhoeae* in Marbrook Chamber and BCS Containing CPBS in Culture Reservoirs. In the last experiment 0.1% CPBS was placed in the culture reservoir and GCB plus soluble starch in the medium reservoir of the Marbrook Chamber, and compared to the Biphasic System containing 100 ml GCA plus 1% Noble Agar, and the Liquid Flask Culture containing 125 ml of only 0.1% CPBS. As indicated by the results recorded in Figure 13, both the BCS and the Marbrook Chamber had an increase in CFU/ml and maintained a high percentage T_2 colony morphology. The Liquid Flask Culture did not maintain growth.

The data from all the experiments were statistically analyzed using a paired t-test. No significant differences (P=0.05) were found between the yields of viable cells under the different growth conditions.

Prosthetic Hemodialysis Culture Unit

The results of the addition of *Neisseria gonorrhoeae* to the PHCU containing 0.85% NaCl in the dialysate culture chamber are graphically depicted in Figures 14 and 15. Initial inoculum was

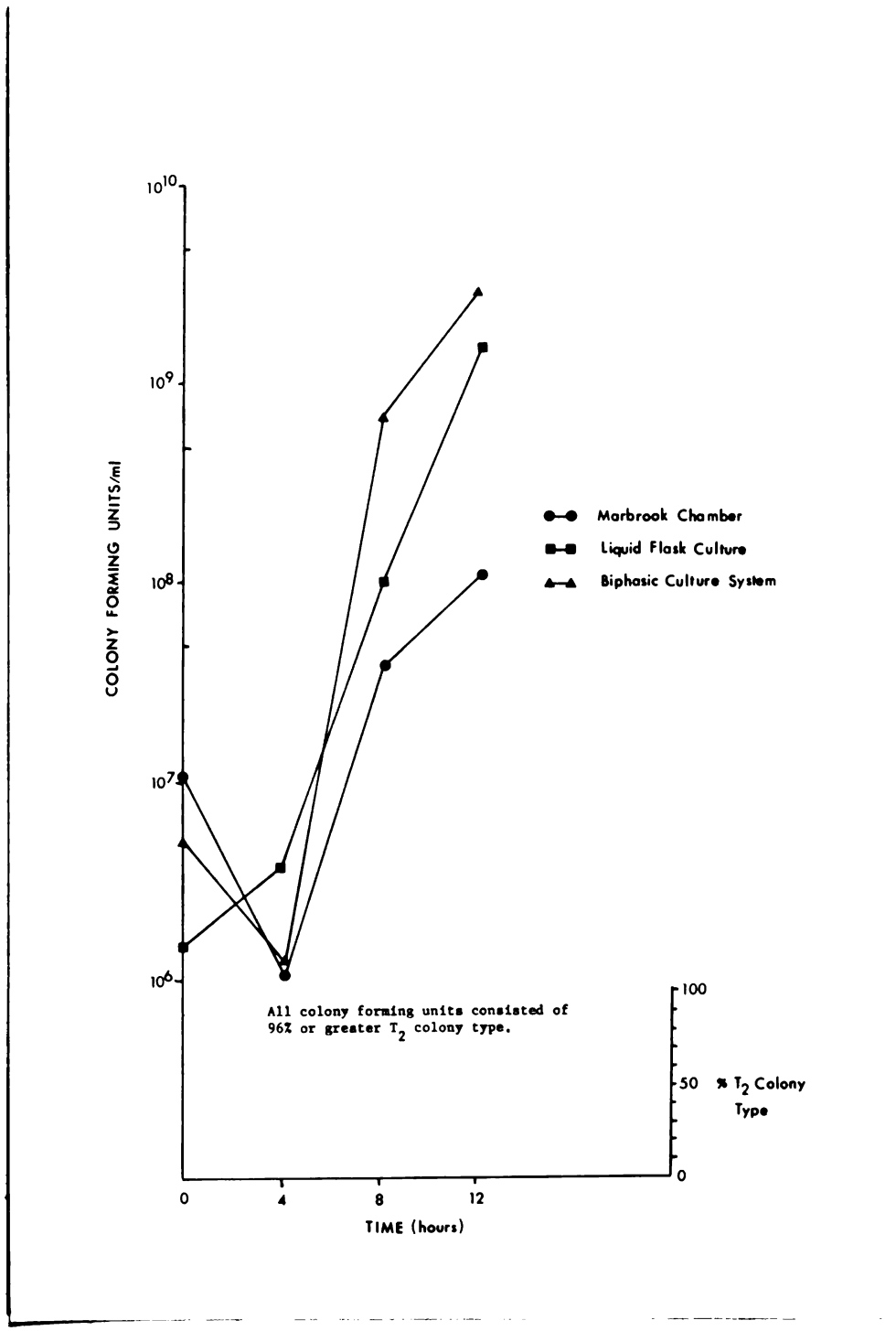

Figure 12. Comparison of the CFU/ml and percent T_2 colony morphology in the Marbrook Chamber, Biphasic Culture System and Liquid Flask Culture.

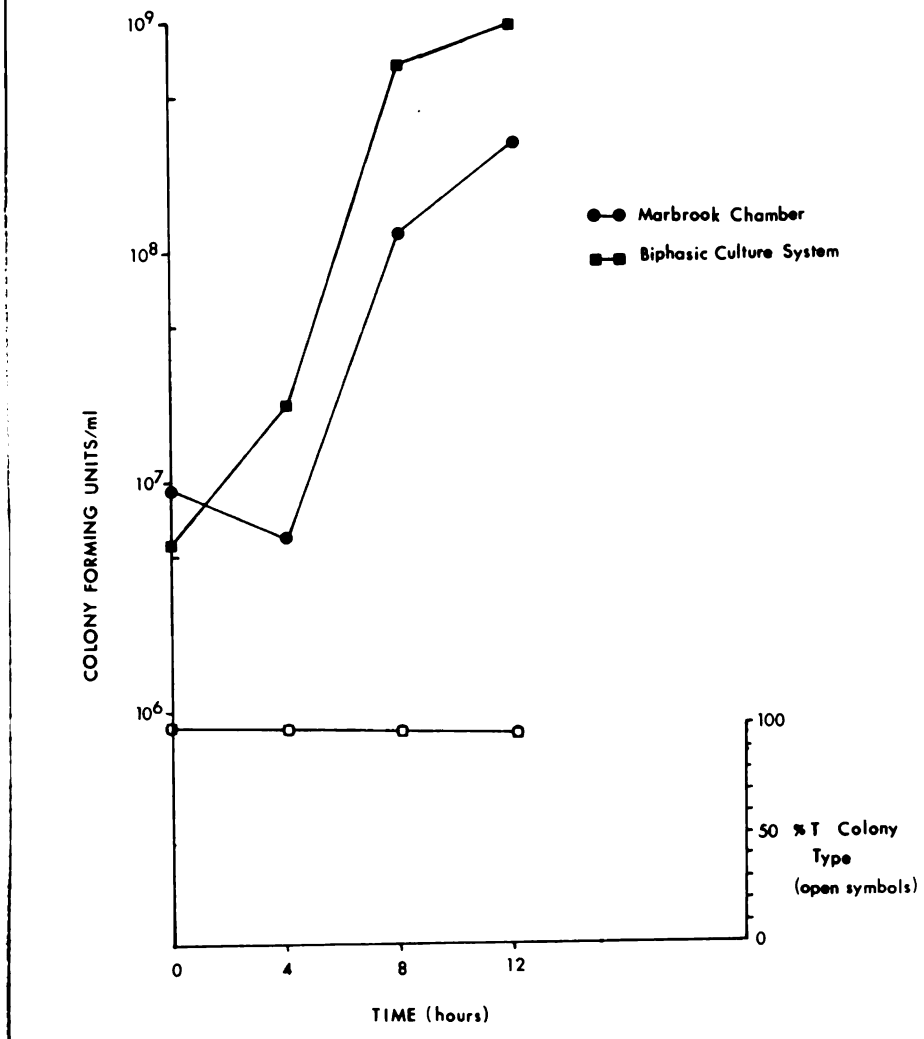

Figure 13. Comparison of CFU/ml and percent T_2 colony morphology in the Marbrook Chamber, Biphasic Culture System and Liquid Flask Culture containing cysteine phosphate buffered saline in culture reservoirs.

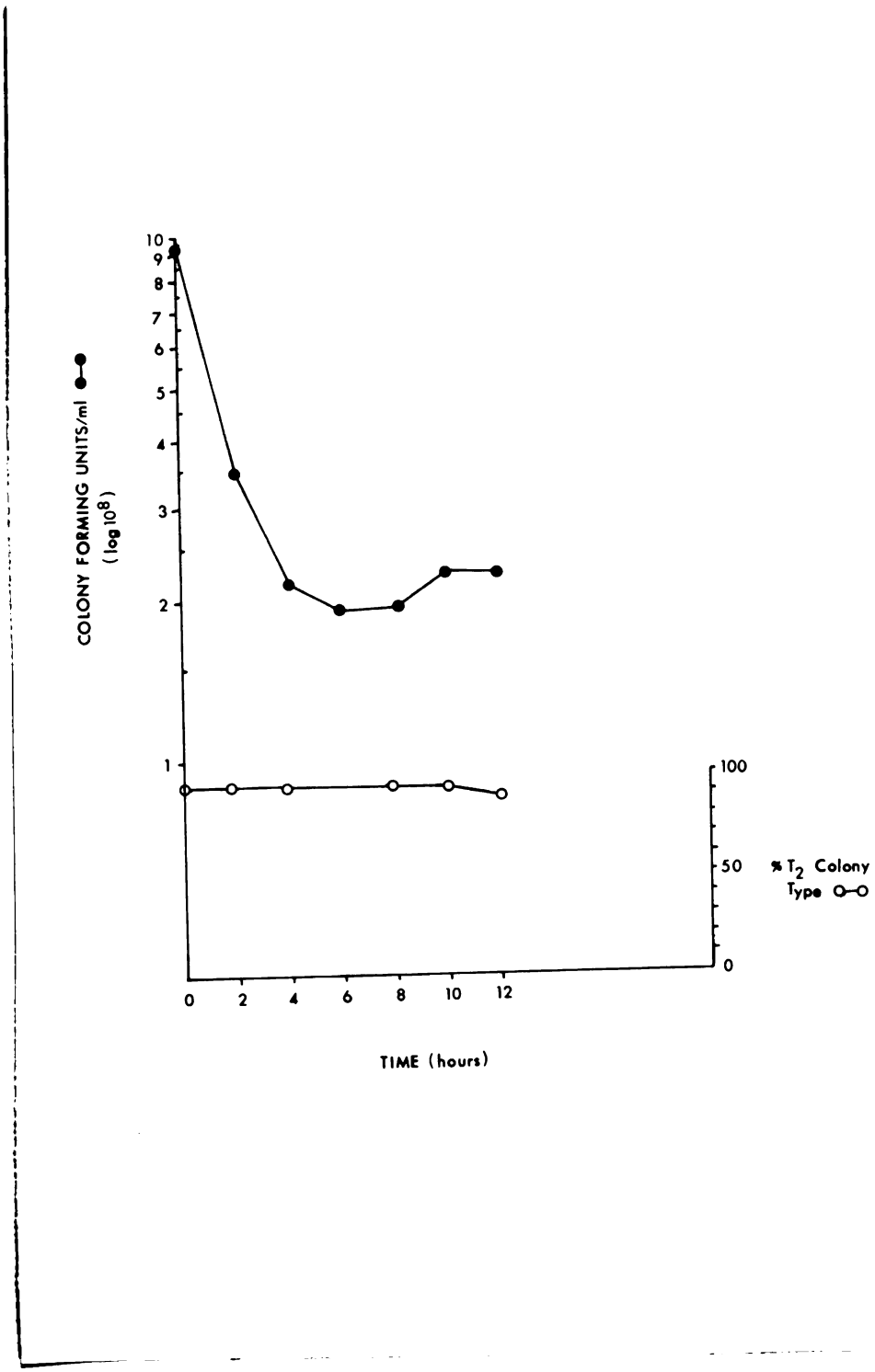

Figure 14. Growth of *Neisseria gonorrhoeae* in the Prosthetic Hemodialysis Culture Unit containing 0.85% saline in the dialysate culture chamber.

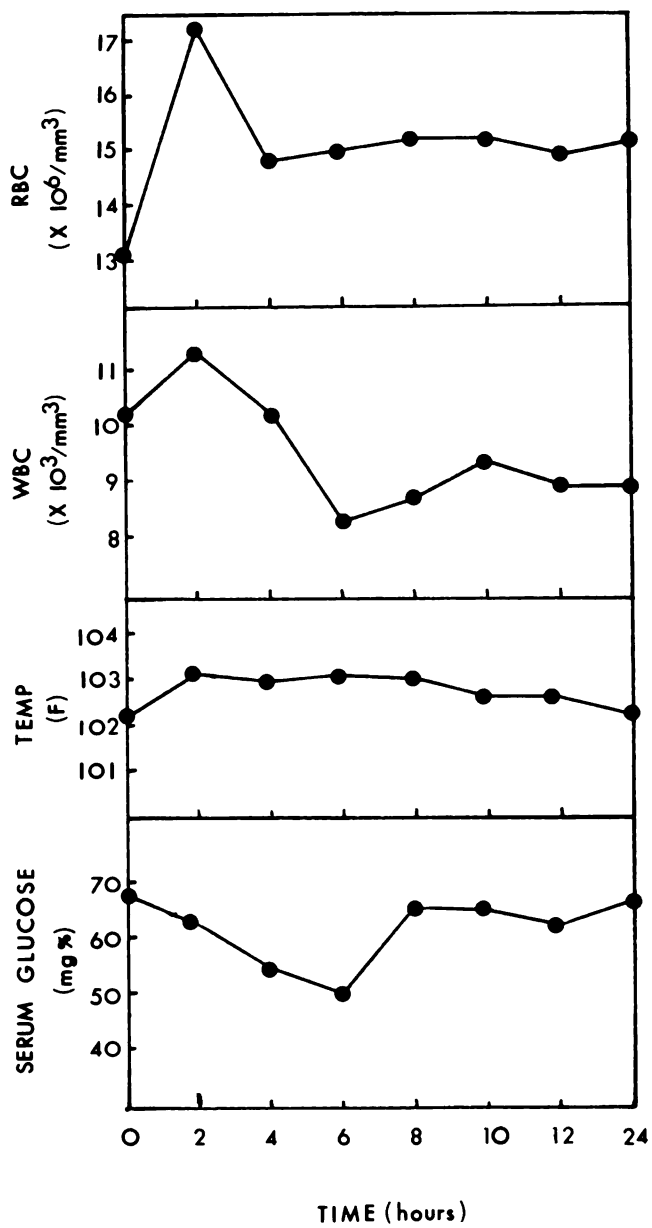

Figure 15. Host response to growth of *Neisseria gonorrhoeae* in the Prosthetic Hemodialysis Culture Unit (0.85% saline).

9.27×10^9 CFU/ml and after 12 h the viable count had decreased to 2.3×10^9 CFU/ml. T_2 colonial morphology was maintained in the 90th percentile for the duration of the experiment. The experiment was terminated at 24 h due to contamination of the dialysate culture chamber. The temperature of the goat remained constant, but the blood glucose gradually dropped over the first 6 h and then rose to previous levels. WBC and RBC counts rose initially and then dropped to a level which was maintained.

When a smaller inoculum was employed (1.69×10^7 CFU/ml) and goat serum was added instead of 0.85% NaCl to the dialysate culture chamber, the cell number decreased to 3.33×10^3 CFU/ml after 2 h and at 4 h no viable cells were detected. Temperature was not recorded as there was no significant change in the previous experiment using a larger inoculum. Serum glucose remained constant. WBC and RBC counts dropped initially and then peaked and declined again (Figure 16).

A viability study comparing *N. gonorrhoeae* in heparinized goat serum and in unheparinized goat serum demonstrated that viability was greater in unheparinized goat serum but was still decreased (Table 5).

The results of the heparin plate dilution sensitivity tests with *N. gonorrhoeae* are depicted in Table 6. After 24 h incubation only the control plate without heparin was countable. The colonies on the plates containing 125 Units (U) and 250 U of heparin/ml were extremely small (0.5 mm) and inhibited; therefore, no attempt was made to count them until 48 h. No growth was detectable on the

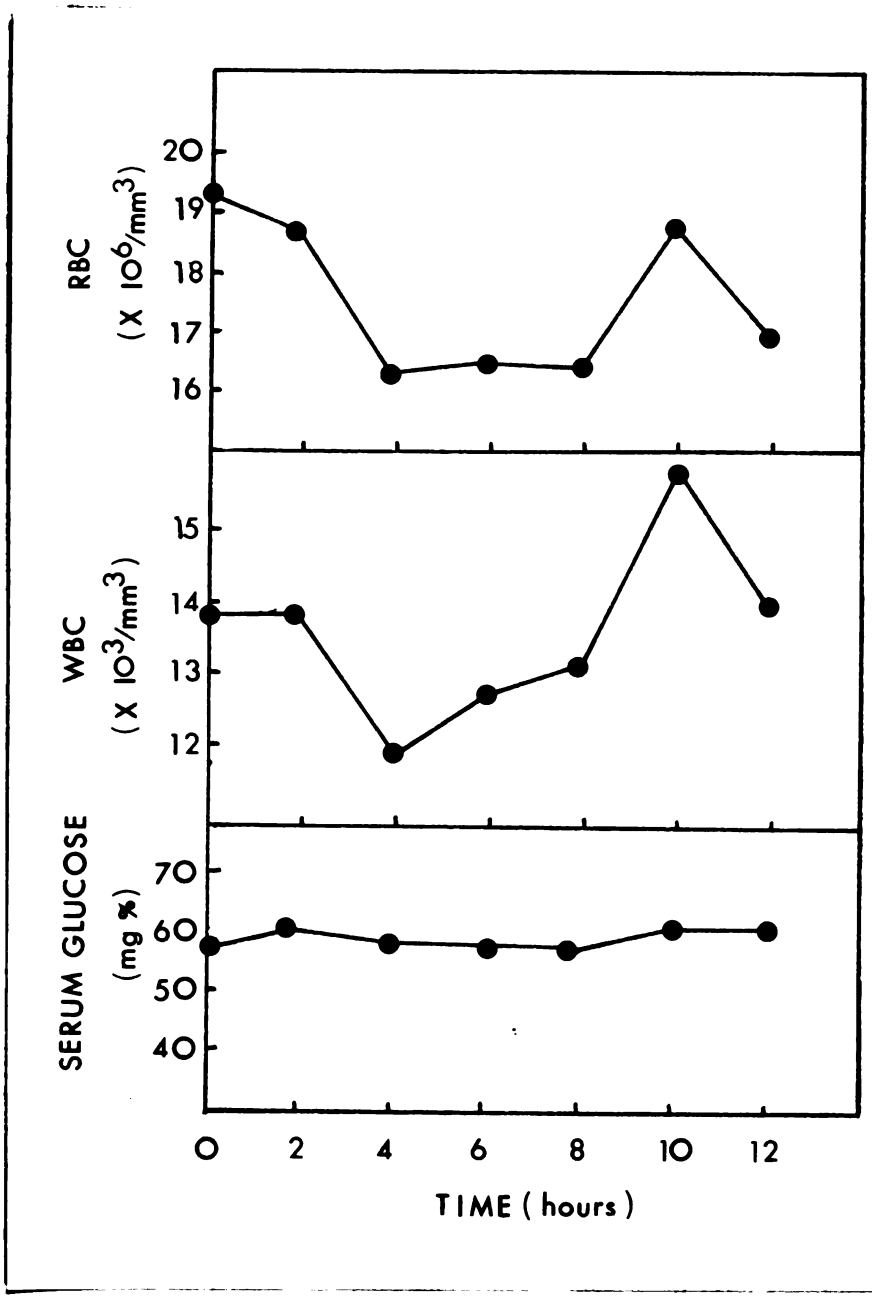

Figure 16. Host response to growth of *Neisseria gonorrhoeae* in the Prosthetic Hemodialysis Culture Unit (goat serum).

Table 5. Effect of heparinized and unheparinized goat serum on *Neisseria gonorrhoeae*

Hours of culture	CFU/ml heparinized goat serum	CFU/ml unheparinized goat serum
0	2.0×10^7	1.46×10^6
2	1.0×10^2	1.43×10^3
12	---	no growth

Table 6. Sensitivity of *Neisseria gonorrhoeae* to heparin using agar plate sensitivity test

Units heparin/ ml GCA	CFU/GCA plate (24 h)	CFU/GCA plate (48 h)
no heparin	121.25	121.25
125	CTSTC[a]	78
250	CTSTC	76.75
500	no growth	28.75[b]

[a]CTSTC = colonies too small to count.

[b]Colonies extremely small; therefore count is a very rough approximation.

plates containing 500 U of heparin/ml after 24 h, and at 48 h a very small number of CFU were detectable but were extremely small and variable in size. It was estimated that the level of heparin in the goat blood was approximately between 250 and 500 U/ml (Dr. Belding, personal communication).

After determining that certain concentrations of heparin had an inhibitory effect on the growth of *N. gonorrhoeae*, the gonococci were exposed to 3 increasing concentrations of heparin in 3 separate Marbrook Chambers and then added to the Prosthetic Hemodialysis Culture Unit. The first Marbrook Chamber contained 1% heparin/ml. The initial inoculum was 7.6×10^8 CFU/ml and after 12 h had increased to 1.7×10^{11}, of which 0.1 ml was transferred to a second Marbrook Chamber containing 10% heparin/ml. At 0 h there were 2.0×10^9 CFU/ml which increased to 6.0×10^{10} CFU/ml in 2 h. One tenth milliliter of this culture was added to the third Marbrook Chamber containing 25% heparin/ml and at 0 h 6.1×10^8 CFU/ml were counted and after 12 h this had increased to 8.1×10^{11} CFU/ml (Table 7).

Table 7. Growth and percent T_2 colony morphology of *Neisseria gonorrhoeae* in increasing concentrations of heparin added to three Marbrook Chambers

Hours of culture	1% heparin/ml		10% heparin/ml		25% heparin/ml	
	CFU/ml	% T_2	CFU/ml	% T_2	CFU/ml	% T_2
0	7.6×10^8	95	2.0×10^9	94	6.1×10^8	90.3
12	1.7×10^{11}	96	6.0×10^{10}	91.7	8.1×10^{11}	89.7

T_2 colony morphology was maintained above 89% in these experiments.

The PHCU was inoculated with 0.3 ml of a 1:1000 dilution taken from the third Marbrook Chamber. The dialysate culture chamber contained 25% heparin/ml in PBS. At 0 h there was 1.36×10^6 CFU/ml in the dialysate culture chamber after which subsequent samples did not

reveal any detectable growth. Host response (glucose, temperature, RBC and WBC counts) was not recorded during this experiment, as it was not deemed necessary based on results of previous experiments.

In the final experiment using the PHCU, a dialysate solution similar to that used in human kidney dialysis was added to the unit and after equilibration for 4 h it was inoculated with 3.67×10^4 CFU/ml. After 6 and 12 h, approximately 3.33×10^3 CFU/ml were detected and after 24 h no growth was detectable. Host response was not recorded.

DISCUSSION

This study provides for a new method for cultivating *Neisseria gonorrhoeae*. Strain 2686, colony type T_2, grew well in the Marbrook Chamber, producing yields of up to 10^{11} CFU/ml. Colony type stability was maintained for 12 h above the 90th percentile. Growth was not significantly different (t-test) from growth obtained using the Biphasic Culture System or Liquid Flask Culture. This could be due to the fact that the clumping or agglutination of T_2 gonococcal cells led to increased sampling inaccuracy and provided only an approximate estimate of CFU/ml. The large aggregates formed probably led to an underestimation of the population of T_2 gonococcal cells. Visual examination of the graphs shows that CFU/ml yield was larger using the BCS than with the Marbrook Chamber. Gerhardt and Gallup (56) did not obtain cell densities with their membrane dialysis flask system as high as those obtained with an agar-liquid system. They postulated that the reason for this was the absence of agar-adsorption in the membrane dialysis system. Some possible problems that might have also caused the decrease in CFU/ml seen in this experiment could have been the adherence of the T_2 gonococci to the dialysis membrane. This could have been compounded by the fact that the momentum of the Marbrook Chamber was less than the momentum obtained in the 250 ml Erlenmeyer flask (due to larger surface

area), even though rotation speed was greater with the Marbrook Chamber.

The medium incorporated in these experiments was a highly enriched complex medium and a much higher yield was obtained in all systems employed when compared with the results of other researchers (76,99).

The stability of colony type T_2 in these experiments was probably due to the fact that the 2686 strain was a stable laboratory strain that had been subcultured many times. The employment of an overnight plate harvest from one T_2 gonococcal colony spread evenly on a GCA plate gave a large and stable inoculum for liquid media.

Agglutination of T_2 gonococci in liquid culture has been mentioned before (76,99) and deterred the use of a spectrophotometer to measure cell density in this study. As mentioned previously, agglutination also leads to some doubt as to statistical relevance of the data. Marquis and Gerhardt (110) reported the use of polymerized salts of sulfonic acids (Daxad 23) to disperse bacterial cells. These agents dissociate in water, into charged anions which adsorb to particles in suspension and impart a negative charge to the particle which results in mutual repulsion. The use of Daxad 23 with *N. gonorrhoeae* seemed to inhibit growth and no dispersion of cells occurred.

Tween 80 (0.02%), like Daxad 23, had no dispersing effect on T_2 gonococcal cells and higher concentrations were quite inhibitory. Catlin (29) did not report any decrease in agglutination with her

complex defined medium, and also did not specify the reason for incorporation of extracted Tween 80.

Another attempt to disperse T_2 cells utilized a decreased NaCl concentration in the medium. This concentration was reportedly used to prepare certain microbial cells (*Streptococcus, Corynebacterium* and *Mycobacterium*) that have a tendency to agglutinate, for serological tests (96). Again no effect on CFU/ml was observed.

Two groups of researchers recently reported success with sonication in dispersing agglutinated T_2 colony forming units. Buchanan and Gottschlich (24) sonicated their T_2 cells for 35 watts/second for 2 seconds. They found that it doubled the CFU/ml and, on microscopic examination, they saw only diplococcal and single coccal forms. Wood and Brownell (191) mildly sonicated T_1 colony type gonococcal cells in an ultrasonic cleaner until a nearly homogeneous diplococcal population was observed, but despite this, clumping developed in their liquid medium. Sonication is often used to disintegrate bacterial cells by the fluctuations of pressure from the wave emission of ultrasonic vibrations (2,149). Alder (2) reported that coccal forms of bacteria are harder to disrupt than rods. This study confirmed the reports of increased CFU/ml with sonication and the reclumping of sonicated cells in liquid broth. Viability apparently was not decreased and colony forming units had the same macroscopic appearance as before sonication. Electron microscopy might be employed to determine if sonication depiliates the T_2 gonococci or alters surface structure in any other way.

In a recent publication by Waldstad *et al.* (176), *Neisseria gonorrhoeae* was found to produce self-inhibitory long chain free

fatty acids and a self-inhibitory phospholipid--monoacylphosphatidyl-ethanolamine. In their experiment they also found that 2% bovine serum albumin added to the agar medium completely blocked these inhibitory substances and that 1% soluble starch partially blocked these inhibitors. Previously it had been thought that the inhibitors of gonococcal growth were in the agar and other components of the medium. Ley and Mueller (103) used a methanol extraction procedure to isolate an inhibitor from agar that was similar in inhibitory effect to steric and oleic acids. Other researchers also reported the inhibitory effect of ordinary agar and recommended the use of purified agar (51,88,90,97).

Bovine serum albumin was found to bind up to 1-2% of its weight in oleic acid (36) and it was presumed to have a greater affinity for fatty acids than other substances. Based on this hypothesis, bovine serum albumin was compared to soluble starch, corn starch and no adsorbant. Experimental data showed little difference between corn starch and soluble starch, but a decrease in CFU/ml was noted using no adsorbant and an even greater inhibitory effect with bovine serum albumin. The discrepancy between results could be due to the difference between the phases in which the albumin is incorporated. It is quite possible that the albumin is more stable in the agar and that if there are toxic breakdown products or components that would have some inhibitory effect on the gonococci, their contact with the gonococci would be increased in liquid media.

Finally, growth was obtained in a clear menstruum in both the Marbrook Chamber and the Biphasic Culture System. Gerhardt and

Hedén (57) reported growth of *N. gonorrhoeae* in a biphasic system of water over complete dextrose-starch agar. The cell yield was lower than was obtained in a complete liquid medium over a complete agar. Results obtained in this study indicated a slight decrease in yield with the Marbrook Chamber in comparison with the BCS, which might be ascribed to reasons mentioned previously. No comparison of growth in the Marbrook Chamber with only CPBS in the culture reservoir to a Marbrook Chamber with a complex medium was made at this time, and a comparison between different experiments is subject to experimental error. The clear menstruum used in these experiments consisted of CPBS instead of water since Hunter and McVeigh (74) reported a decreased viability of cells in inocula prepared in a NaCl solution as opposed to a NaCl-0.1% cysteine solution. Incorporation of phosphate buffers in this experiment helped to stabilize pH. Hunter and McVeigh postulated that the decrease in viability in 0.85% NaCl could be due to the exposure of the gonococci to an adverse oxidation reduction potential. In this experiment, *Neisseria gonorrhoeae* grew in a clear menstruum and thus the enrichments normally incorporated in the medium can be kept separate from the growth environment. In this way cells free from antigenic components of the medium can be obtained. Toxic by-products that limit growth can dialyze out of the culture reservoir and be adsorbed by a compound such as starch, which is incorporated in the medium reservoir. The Marbrook Chamber with its dialysis membrane would eliminate more proteins from the culture reservoir than the BCS in which, due to shaking, proteins would probably break loose from the agar surface and enter the culture broth.

Theoretically some uses for the Marbrook Chamber would be the cultivation of gonococcal cells free from antigenic medium components for the production of vaccines, and the incorporation of labeling compounds used in biochemical and genetic studies which normally are sequestered in the agar when the BCS is used (43). This model might also be expanded so that more surface area and momentum can be gained when the culture is shaken. Two larger membrane dialysis systems are those of Golde (60), who used a stemmed glass bulb covered by a membrane suspended in a 120 ml Erlenmeyer flask, and the twin-chambered dialysis flask designed by Gerhardt and Gallup (56). By increasing the size of the chamber, a higher yield might be obtained.

The *in vivo* part of this study was less successful. The Prosthetic Hemodialysis Culture Chamber designed by Quarles (130) did not support growth of *N. gonorrhoeae*. Starting with a high concentration of gonococci, an initial decrease was noted that later leveled off. When a smaller inoculum was employed the cell population decreased to the point that no growth was detectable. Previously Quarles had been able to grow *Bacillus anthracis, Serratia marcescens, Streptococcus pyogenes, Blastomyces dermatitidis,* and mouse spleen cells, but was unsuccessful with *Treponema pallidum* (130). Considering the remarkable host specificity of *N. gonorrhoeae* it was not surprising that it did not grow in the PHCH. Those organisms that have been propagated thus far in the caprine model are neither especially fastidious in growth requirements or highly specific in their host predilection.

Some of the reasons for the absence of growth of *N. gonorrhoeae* in the unit could have been that the membrane limited nutritional supply to the culture and was poorly permeable by O_2 and CO_2. Quarles discusses these in his thesis as possible explanations for the failure of *Treponema pallidum* to grow (130). It is well known that heparin releases fatty acids in the blood (66,188) and since heparin (M.W. - 6,000 to 20,000) itself was inhibitory in agar plate sensitivity tests it was thought that this could have been the reason for growth inhibition. It was found by gas chromatography that heparinized goat's blood only showed an increase in short chain fatty acids when compared to unheparinized goat's blood (Dr. Beaman, personal communication). It is possible that the direct action of some toxic constituent of goat's blood, e.g., phagocytin, may have inhibited growth. *In vivo* conditions are not fully simulated due to the fact that molecules greater than 10,000 M.W. are excluded by the membrane and the membrane is poorly permeable to gases such as CO_2 and O_2. Quarles (130) found that the dialyzable blood components consisted of sodium, potassium, calcium, magnesium, inorganic phosphate, glucose and urea nitrogen. Future research might employ different types of membranes (nucleopore, etc.) to try and overcome the inhibition.

The Marbrook Chamber might also be of some use in discovering the reason for inhibition of growth in the hemodialysis unit. If growth could be obtained in the latter it would provide a means of studying the host-parasite relationship in bacterial septicemia, *in vitro* versus *in vivo* growth and the effect of antimicrobial drugs.

Other *in vivo* systems such as the coil and "golf ball" models, are inaccessible and hard to sample. They become walled off by adhering macrophages thus limiting diffusion and they are only in secondary communication with the blood (130). Although the Prosthetic Hemodialysis Culture Unit does not simulate *in vivo* conditions fully, it still would have appreciable advantages over the other models.

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