A STUDY OF THE ORGANISMS ASSOCIATED WITH N. GONORRHEA IN MATERIAL FROM PATIENTS

(With Special Reference to Their Effect Upon the Efficiency of Diagnosis by the Culture Method)

by

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(With Special Reference to Their Effect Upon the Efficiency of Diagnosis by the Culture Method)

There are three main types of gonorrhea each of which offers a different problem as far as diagnosis is concerned. There is the early or acute stage with its pronounced clinical signs and purulent discharge which may be diagnosed by the smear technic. Intracellular gram-negative diplococci which resemble the gonococcus, in smears from the male genito-urinary tract, warrant the diagnosis of gonorrhea if the history of the infection or the clinical evidence is presumptive of gonorrhea. In women and children where there are sometimes discharges other than gonorrheal the smear diagnosis alone is less certain and may be misleading.

During the sub-acute and chronic stage, the clinical signs gradually abate to a point of becoming atypical and the exudate may be scanty. Since the gonococci may be few in number their presence on a smear may not be detected. If gram-negative organisms are found on the smear, one cannot be certain about their being N. gonorrhea. Diagnoses of such cases must be confirmed by the culture method.

The third group of cases consists of those persons who may be classified as asymptomatic carriers. The gram-stained smears from these cases may be repeatedly negative, thus

giving much more importance to the culture method for their diagnoses.

DISCUSSION OF PRESENT DIAGNOSTIC METHODS

Smear Method. Only in an acute case with typical clinical symptoms should a diagnosis be made on the basis of a smear alone. The smear method, depending, as it does, solely upon microscopic examination for diagnosis, is an inefficient method when used alone, because there are several bacteria which may closely resemble the gonococcus morphologically. Not only are N. intracellularis and N. catarrhalis indistinguishable morphologically, but other cocci during their stage of division resemble it very closely such as gram-negative forms of streptococci, staphylococci, Diplococcus pneumoniae and coccoidal forms of the colon bacillus. And then, of course, there is always the possibility that gonococci are present on the slide, but are missed by the microscopic examination.

Culture Method. It has long been recognized that the culture method of diagnosing gonorrhea is superior to the gram-stained smear technic. Cohn (1940) of New York in a recent study of vulvo-vaginitis observed that the discrepancy of the smear and culture diagnosis became more and more evident the longer the infection was present. He states that in the advanced state of this disease 40% of the positive cases would be missed by the smear method alone. It has frequently been observed in patients treated with sulfanilamide or its derivatives that the smear report is negative while the culture remains gonococcus positive.

Shaw and Mc Allister (1940) in a series of 136 consecutive cultures taken on cases with a negative gram stain obtained twenty-one positive cultures. Depending upon smears alone one out of five cases would have been misdiagnosed. Carpenter, Leahy, and Wilson (1938) in a study of 245 female patients found cultures positive in 205 (91.9%) and smears positive in only 107 (48%). Cultural methods in their hands proved to be almost twice as reliable as were the microscopic studies of smears.

Even using the culture method, however, false results are sometimes found. There must be some reason for the negative culture results that are sometimes found in positive cases. The organism must have been present in order to have produced the disease, but for some reason failed to grow artificially in the laboratory.

There are at least five possible reasons for the failure of gonococcus colonies to appear when material from positive cases is plated on agar.

1. The organisms are "walled off" within the tissues and are not present in the exudate which is cultured.
2. The specimen was improperly taken.
3. The time that elapsed between the taking of the specimen and the plating out was too long for the survival of the organism.
4. The conditions for growth were not suitable.

(a) Faulty media was used for culturing the organisms.

(b) There was improper incubation as regards temperature, moisture, time incubated, and oxygen tension.

5. The contaminating organisms in the material cultured were so numerous that they produced an inhibitory effect upon the gonococci present.

A brief explanation of each of these points will now be given.

"Walling Off" of Gonococci Within Tissues

If this happens, not only the clinical signs are diminished, but also the number of organisms is decreased. This condition is likely to occur in the sub-acute or chronic stage. These reactions either completely seal off the microorganisms within the tissue for an indefinite time, so that the infection becomes latent, or cause the organisms to be of such a transitory nature, that they will be found intermittently.

The Specimen was Improperly Taken

If the specimen is not properly taken, the organism actually causing the infection can easily be missed, thus leading to false culture reactions. In the male the collection of secretions for cultural studies for gonococci is usually not a difficult matter. One merely has to wash the glans penis with alcohol, to strip the prostate, seminal vesicles and the urethra and have the patient pass immediately thereafter a small quantity of urine into a sterile container.

In the female the story is by no means such a simple one. The following procedures are recommended by the American Neisserian Medical Society (1938).

Bartholin's glands and the urethra, including Skene's glands and their openings, should be inspected and palpated under a good light. The exits of Bartholin's glands should be cleansed, and the glands "milked" to secure a droplet of secretion. The external urinary meatus must also be cleansed. The urethra and Skene's glands are also "milked".

A bi-valve speculum is used to expose the cervix. No lubricant should be used. The external os should be cleansed with dry cotton. The specimen is secured from within the canal with a sterile platinum loop on a glass rod or cotton-wound applicator. A capillary tube is also useful for this. Care should be taken not to carry the infection to the cervix.

Gonococci should be looked for in the rectum by using

a proctoscope. Two smears should be made from each suspected area.

The American Neisserian Medical Society states that culture is the only absolutely certain method of differentiating the gonococcus.

The Time That Elapsed Between the Taking Of the Specimen and the Plating Out Was Too Long For the Survival of the Organism

Although many authors state that the specimens should be placed in the hands of a bacteriologist within a few hours or kept in an ice-box if a longer time is necessary, nothing definite has been found regarding the viability of the gonococcus on the original swab. Since no experimental work was discovered concerning this, we thought that it would be valuable to attempt to find out some data about how long a specimen could be allowed to remain unplated and still show growth of gonococci. Our experimental work on this will be discussed later.

Conditions for Growth Were Not Suitable

Faulty media was used for culturing. As yet we have found no medium that is selective for the gonococcus. We know that it will not grow on ordinary laboratory media, but must be furnished with nutritive material from the body. The p^H of the media is also important, the best growth being obtained in the range p^H 7.2-7.8. The organisms grow much better on media that are freshly made and kept moist.

Bumm in 1885, six years after the organism had been proved to be the etiological agent in gonorrhea, successfully grew the gonococcus on coagulated human blood serum. In 1920 a medium made by Levinthal, which was used at that time for the culture of H. influenzae, was tried. From 1934 to 1938 McLeod and Carpenter experimented with various media in which heated blood was used. These came to be known as chocolate agars. In 1938 Reitzel and Kohl made a plasma hemoglobin agar. Within the last few years particular interest has been taken regarding the best kind of medium to use. In 1931 Pelouze made a calf's brain agar which can now be secured in a powder form from the Digestive Ferments Company, Detroit, Michigan. A few of the newer media that have been tried within the last decade are as follows: Douglas chocolate agar, Bradford's medium, Raven's semi-solid agar, testicular hydrocele agar, ascitic infusion carbohydrate medium, North's gelatin agar, Difco-proteose-hemoglobin agar, plasma-agar medium, and the allantoic membrane of the chick. However,

the subject of a selective medium for the gonococcus needs much more investigation.

Improper incubation. There has long been a difference of opinion regarding the need for air or 10% CO_2 as the better environment for growth. Some claim good results with both methods. Much of this difference of opinion probably is due to the different temperatures at which the cultures were incubated. Growth can be obtained by incubation from 34° C. to 37° C. The optimum temperature appears to be 36° C. Newly isolated strains usually need to be incubated for forty eight hours before visible colony growth appears. If the strain has been transplanted for a long time on artificial media, growth may be obtained after twenty four hours. It is also important that the cultures be kept moist when being incubated. A wet paper towel placed under the petri dishes in the closed container will provide adequate moisture. If any one of these points is neglected, false negative reports may be given out on a positive case.

Contaminating Organisms

Although contaminating organisms of N. gonorrhea are often mentioned by various investigators, very little work has been found by us regarding their isolation and identification by cultural and biochemical reactions. No experimental data has been seen by us concerning the harmful effects, if any, exhibited by these contaminants upon the growth of N. gonorrhea. Therefore, we considered it to be a point worthy of investigation.

The work of Gordon and McLeod (1928) showed that a number of species of bacteria are able to oxidize the compound dimethyl-p-phenylenediamine hydrochloride. Price (1929) used the same compound to test 2,000 cultures made for the gonococcus. He stated that other members of the genus Neisseria also gave a strong oxidase reaction. He also encountered on four occasions a small gram-negative coccus which was evidently not a member of the Neisseria group and which gave a strong positive oxidase reaction. McLeod and his colleagues (1936) stated that in 275 cases, cultures of gram-negative diplococci other than gonococci were obtained in six instances which gave a positive oxidase reaction with the tetramethyl compound.

Thompson (1937) gives a brief discussion about contaminating organisms. In from 1 to 2% of the cultures he encountered organisms which gave a positive oxidase reaction

and which were not gonocccci. In a few instances chromogenic members of the genus Neisseria were found, probably N. flava, and also nonchromogenic, nonfermenting Neisseria, presumably N. catarrhalis. On three or four occasions oxidase-positive yeasts have been found. All of these organisms produce much larger colonies than the gonococcus, and for that reason suspicion is aroused that they aren't gonococci, colonies of which vary between 0.5 and 1.0 mm. in diameter after twenty four hours' incubation.

Thompson states that there are two types of oxidase-positive bacteria which occur in cultures from the genito-urinary tract and which produce colonies of about the same size as that of the gonococcus on original cultures. The first is a small gram-negative rod which grows readily on nutrient agar and which is easily differentiated by its morphologic appearance alone. The second is a gram-negative diplobacillus morphologically, although there is enough variation to make decision difficult as to whether it is a short rod or coccus. Its diameter is within the limits exhibited by the gonococcus. The central portion of the organism appears more lightly stained than the periphery, giving the appearance of a tiny hole. This occurs only in a part of the organisms in a given microscopic field, usually in not more than a quarter or in a half. The distribution of the organism in a stained smear is in masses

or plaques. This is quite different from the relatively even distribution of gonococci in smears from cultures. This organism gives growth on nutrient agar and broth, but it is not profuse. It is non-motile, grows at 25° C., will not liquefy gelatin and ferments glucose, maltose, mannitol, lactose, and sucrose. Thus far it has been impossible to identify definitely these two organisms with species or genera which have been previously described.

Cohn (1940) reports that any unnecessary contamination of the swab or catheter used for the collection of culture material must be avoided. He says that the swab or catheter should come only in contact with the actual place where the exudate appears, otherwise secondary micro-organisms may overgrow the gonococcus. However, no experimental work was given in support of this statement.

PURPOSE OF INVESTIGATION

The purpose of this investigation is to find out if the presence of contaminating organisms has anything to do with the negative results found in certain positive cases. Since we almost never are dealing with a pure culture of gonococci when obtaining a specimen from a case of gonorrhea, we must consider that the growth of the gonococci present might be different when plated on an agar plate along with many contaminating organisms than it was when growing probably in pure culture within the human tissue. Although it may not be possible to obtain the organism in an uncontaminated state, we are attempting to see if greater care need be taken to obtain the specimen as free from contaminants as possible.

If by studying the effects of these various organisms separately and in different combinations upon the growth of the gonococcus, we can show that a higher percentage of positive cases can be found if these contaminants are eliminated, a contribution will be made to the culture method of diagnosing cases of gonorrhea.

If it is found by experimentation that the gonococci grow better when not contaminated, then we must assume that the extraneous organisms present have an inhibitory effect upon the growth of the gonococci. To be of any practical importance this must be more of an inhibition than that

which would result merely from the presence of a large number of organisms on the plate. We have observed, for example, that if a large number of N. gonorrhea colonies are present on a plate, the colonies will be smaller than if there were only a few isolated colonies spread about the plate.

EXPERIMENTAL WORK

Materials

All the specimens used for this work were from women patients, and were obtained by Dr. Miriam Luten from the Multnomah County Clinic. At first various media were used in growing pure cultures of gonococci. Those media used the most were: sheep testicular ascitic agar, North's gelatin agar, Difco proteose hemoglobin agar, and Douglas agar, all of which gave good results. However, since Douglas chocolate agar, made by adding from five to ten percent of fresh blood to Douglas agar when at 85° C., is probably used the most widely and since all the plated specimens received were on this agar, it was thought advisable to use this as a standard medium. Therefore, all the experiments done involving N. gonorrhea employed the use of Douglas chocolate agar.

Methods

Various strains of gonococci were isolated from Douglas chocolate agar plates which had been incubated at 36° C. in 10% CO_2 for forty eight hours. The gross appearance of the gonococcus colonies, the microscopic and staining characteristics, and the sugar reactions were determined. In connection with the last named observation a brief study of various indicators thought to be suitable was made. The indicators found to give the best color changes with glucose media and gonococci were cresol red, changing color through a p^H range of 7.2 to 8.8 and phenol red with a range of color change of p^H 6.8 to 8.4. Phenol red was used in a final concentration of 0.0005%, obtained by adding twenty five cubic centimeters of a 0.02% aqueous solution of the indicator to a liter of the medium.

All the streptococci which were taken from the plates were inoculated into Hartley broth and incubated for twenty four hours. Blood plates of these organisms were made to find out whether or not the streptococci were hemolytic. The pure cultures were then incubated on blood slants and after twenty four hours incubation were kept in the ice-box. A few of the strains of streptococci were classified by Lancefield's classification.

All the other contaminants were inoculated on infusion agar slants. When good growth was obtained, a gram stain

was made to find out if the culture was a pure one and to study the morphology. Fermentation studies were made of all the contaminants on glucose, sucrose, and lactose broth tubes. The microscopic appearance of the contaminating gram-positive bacilli and the gram-negative rods was noted. Gelatin liquefaction and coagulase tests were run on all the staphylococci as an aid toward finding which ones were pathogenic. Blood plates were streaked with each contaminant for the purpose of determining the hemolytic properties, pigment formation, and oxidase reactions.

Contaminants

Although many of the cultural reactions of the contaminating organisms were studied, in some cases, especially in regard to the gram-positive bacilli and the streptococci, it was impossible to identify the organism by name without spending more time on the problem than seemed practical. All the contaminants which were mixed with the gonococci in the following experiments were those isolated from actual cases of gonorrhea.

Many contaminating colonies were picked from each Douglas chocolate agar plate on which a specimen had been plated. It was hoped that by this means all the different types of bacteria occurring on each plate would be found. Many colonies picked from one plate on identification turned out to be of the same species as others and some types were probably missed. But in general it seems probable that the collection ultimately obtained represented the most common organisms associated with N. gonorrhea in specimens taken from patients. Some of the plates examined turned out to be negative for N. gonorrhea. Therefore, the organisms picked from these plates represent the organisms most commonly found in suspected cases of gonorrhea. Specimens from both the cervix and the urethra were examined.

The characteristics of all the staphylococci picked

from the plates are summarized in Charts 1, 2, 3, 4, 5, and 6. From the plates are summarized it can be seen that specimens from thirteen positive gonorrhea cases showed the presence of various kinds of staphylococci. Eight specimens negative for N. gonorrhea contained staphylococci. Although there is no certain laboratory test for determining the pathogenicity of staphylococci, the various tests which are usually done for testing this property are gelatin liquefaction, hemolysis on blood plates, pigment formation, and coagulase tests. The first three tests mentioned do not correlate as well with pathogenicity as the coagulase test does. Staphylococci from twenty four patients gave positive coagulase tests, indicating that they were pathogenic strains.

Charts 7 and 8 summarize some of the characteristics of the gram-positive bacilli found. More work, however, needs to be done on the classification of these organisms.

The results found on the yeast organisms seen on Chart 10 are especially interesting since these organisms give positive oxidase reactions. Although the colonies do not resemble those of N. gonorrhea, they might be confusing to some in that they give a definite black color with tetramethyl-p-phenylene-diamine hydrochloride.

Gram-negative bacilli were found in several cases, B. coli being identified in specimens from just one patient.

From a patient named Allen was isolated a gram-negative rod, whose characteristics are seen on Chart 9. It is a tiny gram-negative bacillus that was not identified by us. It occurs frequently on the N. gonorrhea plates, and is confusing because the colony is colorless, pin-point, and oxidase positive. Evidently it is not H. influenzae since it does notdemonstrate the satellite phenomenon on blood plates.

Streptococci were isolated and identified by Lancefield's classification from five cases. Four of these strains were identified as belonging to species Streptococcus pyogenes, groups A and G, both of which are considered to be pathogenic for man. A hemolytic streptococcus was isolated from the eye of one patient thought possibly to be a case of ophthalmia neonatorum. Although the organism grew on extract agar and was, therefore, considered at first to belong to the Enterococci group of streptococci, it was later proved to be Type A of the pyogenic streptococci. These results are shown on Chart 11.

Chart 1

Staphylococci

Specimen	Color-Size of Colony	Glucose	Sucrose	Lactose	Gelatin	Blood Hemolysis	Pigment on Blood	Coagulase	Oxidase
Alexander	white 1 mm.	+	+	+	−	−	−	++++	−
"	orange .2 mm.	+	+	+	+	−	+	+++	−
Davidson	white 1 mm.	+	+	+	−	−	−	+++	−
Bauholeman	white .5 mm.	−	−	−	−	−	Slight amount	+	Bright blue
"	white 1 mm.	−	−	−	−	−	−	++	−
Neil *	white 1 mm.	−	−	−	−	−	−	+++	Bright blue
Cogger *	bright yellow 1 mm.	−	−	−	−	−	white, gram-neg. yellow, gram-pos.	++	Bright blue
Smith *	white 1 mm.	−	−	−	−	−	−	−	−
Ernst *	yellow .5 mm.	−	−	−	−	−	−	−	−

Chart 2

Staphylococci

Specimen	Color-Size of Colony	Glucose	Sucrose	Lactose	Gelatin	Blood Hemolysis	Pigment on Blood	Coagulase	Oxidase
Ernst *	white .5 mm.	+	+	+	—	—	—	+	—
Brant *	opaque white .5 mm.	—	—	—	—	—	—	+	—
Williams *	yellow 1 mm.	+	—	—	—	—	+	—	—
"	yellow .5 mm.	—	—	—	—	—	—	++	—
"	blue white 1.5 mm.	—	—	—	+	+	—	—	—
Crawford	blue white 2 mm.	+	—	—	+	+	—	—	—
"	opaque white 1 mm.	+	+	+	—	—	—	+	—
"	white pin-point	+	—	—	+	—	+	+	—

Chart 3

Staphylococci

Specimen	Color-Size of Colony	Glucose	Sucrose	Lactose	Gelatin	Blood Hemolysis	Pigment on Blood	Coagulase	Oxidase
Crawford	opaque white pin-point	+	+	+	−	−	+	−	−
"	pale yellow 1 mm.	+	+	−	−	−	+	−	−
"	gray .5 mm.	+	+	+	+++	+	−	−	−
Chalk *	white 2 mm.	+	+	+	+++	−	−	−	−
Allen	white pin-point	+	+	−	+	+	−	+	−
Austin	bright orange	⊕	−	+	−	−	+	+	−
"	white .5 mm.	+	+	+	+++	−	−	−	−
Clark	soft yellow 1 mm.	+	−	−	++	−	+	+	Bright blue

Chart 4

Staphylococci

Specimen	Color-Size of Colony	Glucose	Sucrose	Lactose	Gelatin	Blood Hemolysis	Pigment on Blood	Coagulase	Oxidase
Clark	gray, spreading 2 mm.	+	+	+	++	—	—	—	—
"	opaque white 1 mm.	+	+	+	++	—	—	+	—
"	soft white 2 mm.	+	+	+	—	—	—	—	—
Bauer *	opaque white 1 mm.	+	+	+	+++	—	—	+	—
Palmer *	white .3 mm.	+	+	+	+++	—	+	—	—
"	white .2 mm.	+	+	+	—	—	—	—	—
"	bright yellow, raised. .5 mm.	—	+	—	++	—	Bright orange	—	—

Chart 5

Staphylococci

Specimen	Color-Size of Colony	Glucose	Sucrose	Lactose	Gelatin	Blood Hemolysis	Pigment on Blood	Coagulase	Oxidase
Palmer *	opaque white 1 mm.	+	+	+	−	+	−	+	−
"	gray .5 mm.	+	−	−	−	−	−	−	−
"	spreading yellow 2 mm.	+	+	+	+++	−	+	+++	−
Preston	colorless, looks like gonococcus .3 mm.	+	−	+	−	−	−	−	+
Nelson *	white 1 mm.	+	+	−	+	−	−	−	−
"	creamy white 1 mm.	+	+	+	++	−	−	−	−
"	orange .5 mm.	+	+	−	−	−	+	+	−

Chart 6

Staphylococci

Specimen	Color-Size of Colony	Glucose	Sucrose	Lactose	Gelatin	Blood Hemolysis	Pigment on Blood	Coagulase	Oxidase
* Nelson	cream .5 mm.	+	+	+	+	+	—	++	—
"	opaque white pin-point	+	+	+	+	—	—		—
* Moore	white 2 mm.	+	+	+	+	Large amount	—	+	—
"	white 1 mm.	+	+	+	++	—	—	—	—
Brem	gray-white .5 mm.	+	+	+	++	—	—		—
French	gray-white .5 mm.	—	—	—	—	—	—		—

Symbols: * Positive gonorrhea case.

Chart 7

Gram-positive bacilli

Specimen	Color-Size of Colony	Microscopic Appearance	Glucose	Sucrose	Lactose	Oxidase
Davidson	crinkled-rough, white colony	Large bacilli	+	+	+	—
* Bauholeman	white .2 mm.	cigar-shaped bacilli	—	—	—	Bright blue
* Cogger	white	long-chained bacilli	—	—	—	—
"	bright yellow	"	—	—	—	—
* Brant	yellow colony	bacilli	+	+	—	—
Crawford	white with rough edges	short, plump rods	—	—	—	—
* Korman	white	bacilli	+	—	—	—
* Pinchney	white .5 mm.	pleomorphic short plump bacilli	+	—	—	—
"	colorless 1 mm.	"	+	+	—	—
* Chalk	white	bacilli	—	—	—	—

Chart 8

Gram-positive bacilli

Specimen	Color-Size of Colony	Microscopic Appearance	Glucose	Sucrose	Lactose	Oxidase
Crawford	cream-white 1 mm.	pleomorphic rods	+	—	—	—
Palmer *	white, uneven edges 1 mm.	"	+	—	—	—
"	opaque white, rough edges 1 mm.	"	—	—	—	—
Bauer *	white .5 mm.	"	+	—	—	—
"	gray-white 2 mm.	club-shapes	+	+	—	—
"	white .5 mm.	tiny bacilli, staph. formation	+	+	—	—

Symbols: * Positive gonorrhea case.

Chart 9

Gram-negative bacilli

Specimen	Color-Size of Colony	Microscopic Appearance	Glucose	Sucrose	Lactose	Oxidase
Crawford	mucoid, colorless	long rods	–	–	–	–
Hayes	colorless .5 mm.	short rods	⊕	–	⊕	–
Clark	opaque white .5 mm.	"	–	–	–	bright blue
Allen	colorless, pin-point	tiny rods- resemble H. influenzae	–	–	–	+

Chart 10

Miscellaneous organisms

Specimen	Color-Size of Colony	Microscopic Appearance	Gram Stain	Glucose	Sucrose	Lactose	Pigment	Hemolysis	Oxidase
Bauer	opaque white 1 mm.	budding yeast	+	⊕	+	−	−	−	+
"	gray-white .5 mm.	"	+	⊕	+	−	−		+
King	large, mucoid	large, spore-forming bacillus.	+	+	+	−	−	−	−
Landbaum	white .5 mm.	large, diplococcus. N. catarrhalis	−	−	−	−	−	−	+

Chart 11

Streptococci

Specimen	Lancefield Group	Hemolysis	Fibrinolysis	Division	Species
Ernst *	A	+	+ +	Pyogenic	Strep. zymogenes
Korman *	G	+	+	Pyogenic	Group "G"
Crawford		+		Enterococci	Strep. zymogenes
Landbaum	A	Large am't.		Pyogenic	Strep. pyogenes

Symbol: * Positive gonorrhea case.

Inhibiting Effects of Contaminants On N. gonorrhea In Broth Culture Mixtures

Addition of a constant amount of a contaminating organism to varying dilutions of the gonococci. It was thought that any inhibiting effects upon the growth of N. gonorrhea could be discovered if plates were made from a series of dilutions of a pure culture of gonococci and a similar series of contaminated cultures. To each dilution of gonococci comprising the contaminated series was added a constant amount of a contaminant whose cultural characteristics were known.

The dilutions of gonococci were carried far enough so that there was no growth of gonococci in the highest dilutions of the uncontaminated series. This series served as a control. From each dilution one drop was plated out on Douglas chocolate agar. This was spread around on the plate with a sterile glass spreader. All the plates were inoculated as soon as the mixtures were made and were incubated at 36° C. for forty eight hours in an atmosphere containing ten percent CO_2. The results of one such experiment are given in Table 1 from which it appears that the gram-positive bacilli must have produced a deleterious effect upon the gonococci, since the growth of the latter appeared in higher dilutions on the control plates than on the contaminated ones.

Table 1

Inhibition of Growth of N. gonorrhea by a Gram-Positive Bacillus				
	First Dilution	Second Dilution	Third Dilution	Fourth Dilution
Control Plates	Pin-point colonies of gonococci. Entire plate covered.	Large isolated gonococcus colonies.	Six gonococcus colonies. 1.5 mm. in diameter	No gonococci present.
Contaminated Plates	Plate covered with diptheroids A few gonococci.	No gonococci.	No gonococci.	No gonococci.
Symbols: * Highest dilution where growth of gonococci was seen on the plates.				

Similar experiments were carried out to test the harmful effects of other organisms commonly found associated with the gonococcus. Five dilutions of a suspension of N. gonorrhea were made using infusion broth in five cubic centimeter portions. In all the dilutions one cubic centimeter of broth was transferred to the next tube. Five control plates on Douglas chocolate agar were made from these. After these plates were made, to each tube were added two loopfuls of a staphylococcus suspension. Three more series of gonococci suspensions exactly the same as the control series were made. To each tube of one of these series were added two loopfuls of a streptococcus suspension. Likewise, B. subtilis and a gram-positive diptheroid were added to the other two series of suspensions. One plate was made from each of these suspensions.

All the plates from these mixtures were made at the same time and were all incubated at 36° C. in ten percent CO_2 for forty eight hours before being examined for the presence of oxidase positive colonies. Table 2 gives the results of these tests.

This data gives evidence pointing to the fact that the gonococci will grow more abundantly on a plate that is free from other organisms. On all the control plates of pure gonococci made from the first three dilutions N. gonorrhea colonies appeared. N. gonorrhea colonies appeared on only

Table 2

Effects of staphylococci, streptococci, B. subtilis, and a Gram-Positive Bacillus on the Growth of N. gonorrhea

	First Dilution	Second Dilution	Third Dilution	Fourth Dilution
Controls Pure cultures of gonococci.	20 gonococcus colonies.	8 gonococcus colonies.	5 gonococcus colonies. *	No gonococci.
N. gonorrhea plus staphylococci.	10 gonococcus colonies.	5 gonococcus colonies. *	No gonococci.	No gonococci.
N. gonorrhea plus streptococci.	18 gonococcus colonies.	3 gonococcus colonies. *	No gonococci.	No gonococci.
N. gonorrhea plus B. subtilis.	Many contaminants. No gonococci. #	No gonococci.	No gonococci.	No gonococci.
N. gonorrhea plus a gram-positive bacillus.	15 gonococcus colonies.	2 gonococcus colonies. *	No gonococci.	No gonococci.

Symbols: * Highest dilution where growth of gonococci was seen on the plates.

\# The plate was entirely spread over by B. subtilis, preventing the detection of any oxidase positive colonies.

the plates made from the first and second dilutions of gonococci when staphylococci were added. Likewise, N. gonorrhea colonies appeared from only the first and second dilutions of gonococci when mixed with a gram-positive bacillus. One gonococcus colony appeared from the third dilution of gonococci when streptococci were added, while there were five oxidase positive colonies on the control plate of the same dilution. No gonococci appeared on any of the plates inoculated with the mixture of B. subtilis and N. gonorrhea.

The results of a third experiment, similar in nature, demonstrate also that gonococci are inhibited to a large extent by the presence of contaminating organisms.

Eight dilutions using Hartley broth of a suspension of N. gonorrhea were made from a twenty four hour pure culture. Plates were made of Douglas chocolate agar and two drops from dilutions 1,2,3,5, and 7 were inoculated on these plates. These were used as controls. Suspensions of a gram-positive staphylococcus and a gram-positive bacillus were made. Three drops of the staphylococcus suspension were then put into each tube of the control dilution series after the controls had been plated. A fresh series of gonococci containing the same number of organisms as the control tubes was made up and to each tube were added three drops of a suspension of a gram-

positive bacillus. All the plates were inoculated with two drops of the suspensions. Table 3 gives the results of this experiment.

In this experiment the dilutions of gonococci weren't carried far enough so as to entirely exclude the growth of the gonococci on the control plates. Nevertheless, there is a distinct difference between the amount of the growth of N. gonorrhea on the control plates and on the contaminated plates. Although the same amount of a suspension of gonococci was used in all cases, the plates containing the contaminants showed a marked decrease in the growth of N. gonorrhea. This inhibition couldn't have been due to the fact that the contaminants caused the plate to be too crowded for growth, leaving little room for the gonococci, since there was room for many more colonies on the plate.

Table 3

Inhibition of the Growth of N. gonorrhea by staphylococci and Gram-Positive Bacilli			
	Controls-Pure culture of N. gonorrhea.	N. gonorrhea plus staphylococci.	N. gonorrhea plus a gram-positive bacillus.
First Dilution	Many gonococcus colonies. Plate crowded. Colonies 0.5 mm. in diameter.	Plate crowded with staph. and gonococci. Pin-point colonies.	Plate crowded with gonococci and contaminants.
Second Dilution	Plate crowded with gonococcus colonies.	Many staphylococci. Many pin-point gonococci.	Plate crowded with gonococci and contaminants. Many oxidase positive pin-point colonies.
Third Dilution	Plate crowded with gonococcus colonies.	Many staphylococci. Pin-point gonococci.	Many large gonococcus colonies 1.0 mm. in diameter.
Fifth Dilution	About 200 isolated oxidase positive colonies 1.0 mm. in diameter.	Many staphylococci. Few gonococci.	5 gonococcus colonies seen.
Seventh Dilution	About 100 isolated gonococcus colonies 1.0 mm. in diameter.	Many staphylococci. Few gonococci.	2 oxidase positive colonies seen. Many contaminants.

Pure broth cultures of gonococci of the same dilutions and contaminants were allowed to remain together as mixtures for various times and temperatures before being plated out. In the above experiments the mixtures of gonococci and the contaminants were plated out as soon as they were mixed, but the following work shows the effects of various organisms upon N. gonorrhea after the mixtures have been kept for varying intervals and at different temperatures before being plated out. In the former the number of gonococci used was the variable factor; in the latter, the time that the gonococci remained together with a contaminant in a broth culture before being plated out was the variable factor.

At the beginning of these experiments all the mixtures contained the same number of gonococci and contaminants. If the gonococci appeared on the plates made from the uncontaminated control plates while they failed to appear on the corresponding contaminated plates, then we assumed that the contaminating organisms must have had an inhibiting effect on N. gonorrhea.

An experiment involving N. gonorrhea mixed separately with strains of streptococci, B. coli, Staphlococcus aureus, and a gram-positive bacillus was run for thirty hours at room temperature to see if there was any inhibition of the growth of the gonococci and how long the organisms would

survive contaminated and uncontaminated. All the broth tubes remained at room temperature during the experiment. Although the plates were made every three hours for thirty consecutive hours, there was no growth of gonococci on any of the plates after twelve hours.

The gonococci on the control plates grew for twelve hours in a broth tube standing at room temperature. This was a longer time than that for any of the contaminated gonococci. On those plates mixed with B. coli where the growth of B. coli was very heavy no gonococci appeared on any of the plates although the same amount of a live gonococcus culture was used. Evidence, therefore, points to the fact that the contaminants must have had an inhibiting effect upon the growth of the gonococci. Details of this experiment are seen in Table 4.

Figure 1, derived from data in Table 4, shows three death curves of N. gonorrhea. One curve shows approximately the rate of destruction of a pure culture of N. gonorrhea while remaining in a broth tube at room temperature over a period of twelve hours. In all three cases there were the same number of gonococci at the start of the experiment. Another curve representing a mixture of gonococci and streptococci shows that a smaller number of gonococci appeared on the first plate than did on the first plate made from the uncontaminated culture. If the two curves

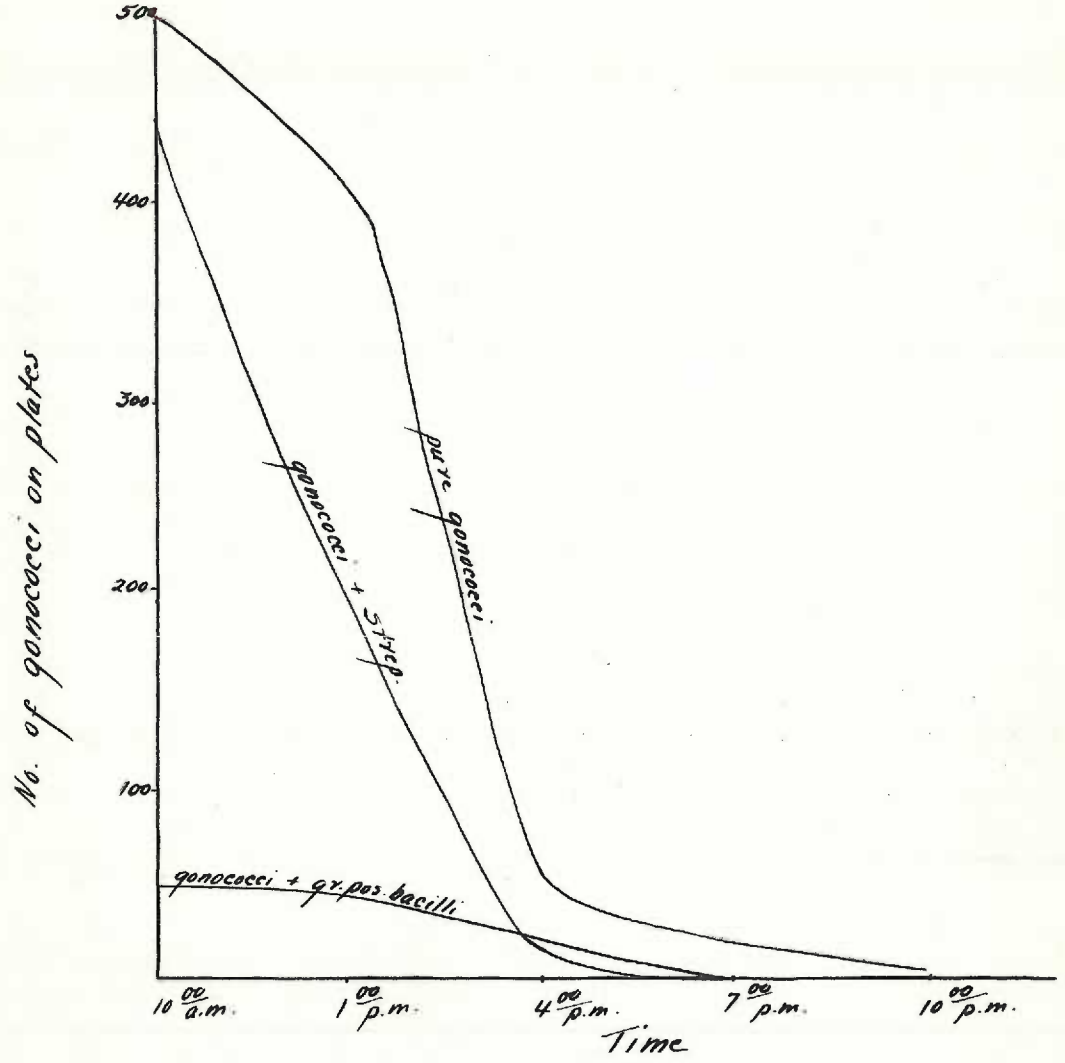
Table 4

Growth of Gonococci in Broth with Contaminating Organisms at Room Temperature					
	10:00 a. m.	1:00 p. m.	4:00 p. m.	7:00 p. m.	10:00 p. m.
Controls- Pure culture of N. gonorrhea	Plate crowded with gonococci	Plate evenly covered with gonococcus colonies	40 large gonococcus colonies	20 gonococcus colonies	2 gonococcus colonies *
Streptococci plus gonococci	Plate covered with gonococci	200 gonococcus colonies	4 gonococcus colonies *	No gonococci	No gonococci
B. coli plus gonococci * #	No gonococci- Plate covered with B. coli	No gonococci	No gonococci	No gonococci	No gonococci
Staph. aureus plus gonococci	7 gonococcus colonies *	No gonococci	No gonococci	No gonococci	No gonococci
Gram-positive bacilli plus gonococci	40 gonococcus colonies	40 gonococcus colonies	6 large, 25 small gonococcus colonies *	No gonococci	No gonococci

Symbols: * Highest dilution where growth of gonococci was seen on the plates

\# The plate was entirely spread over by B. coli, entirely inhibiting the growth of N. gonorrhea.

Figure 1



Death Curves of Gonococci Kept at Room Temperature With and Without Contaminating Organisms

are compared, it will be seen that there is a sharper decrease in the number of gonococci in the contaminated tube. The third curve shows that there were very few gonococci on any of the plates made from the mixture of gonococci and gram-positive bacilli. The death curve here appears to be more gradual in comparison to the others.

A suspension of a recently isolated strain of N. gonorrhea was made with Hartley broth. This was divided into four portions. To two portions was added a loopful of staphylococci from a twenty four hour glucose broth tube. One control tube containing only gonococci and one contaminated tube were left at room temperature and plated out at intervals. The two other tubes were kept in the incubator and plated at the same times. One drop of each suspension was made on each plate and spread with a glass rod. The suspensions were made at 9:00 a. m., the first plates were made at 10:00 a.mm., and the last ones were made at 6:00 p. m. of the same day. Table 5 and Figure 2 give the results of this experiment.

Since the gonococci grew longer on the control plates made from uncontaminated suspensions kept at room temperature than on any of the other plates, we might say that the growth of the gonococci was arrested by the following factors:

(a) Standing at room temperature for many hours before being plated out.

(b) Remaining at incubator temperature as a pure

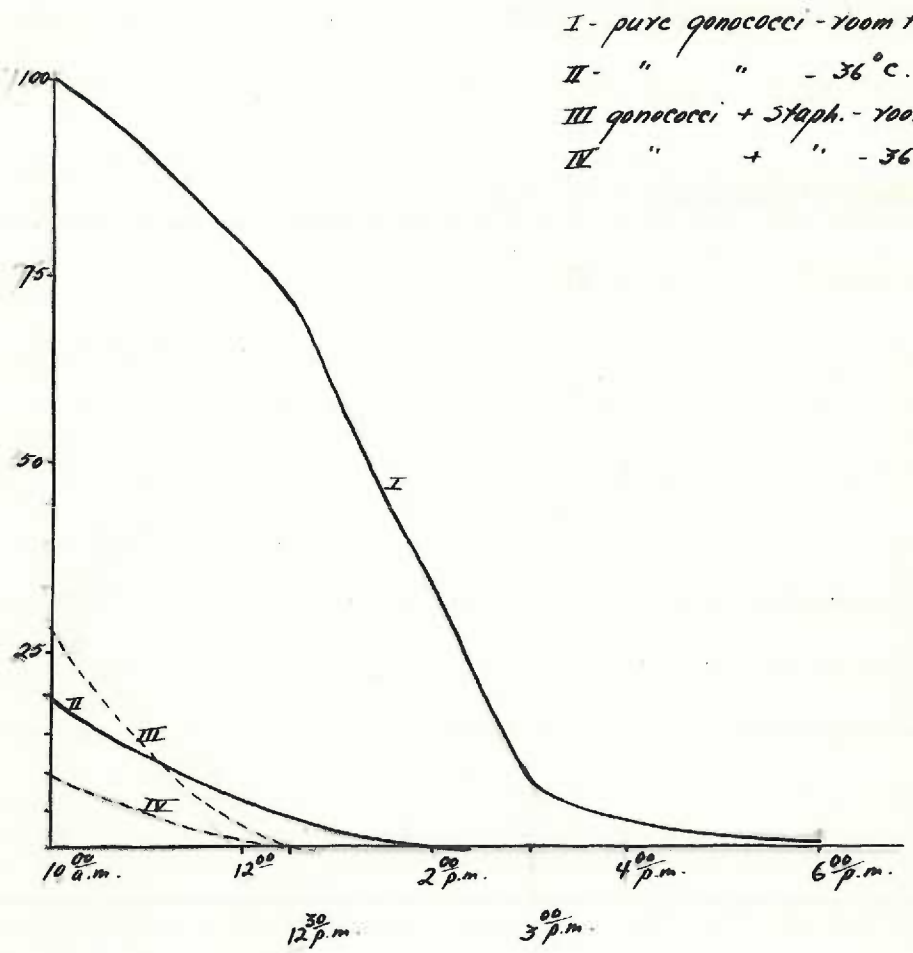
Table 5

A Comparison of the Inhibition of the Growth of N. gonorrhea by Contaminating Organisms at Room Temperature and at 36° C.

	10:00 a. m.	12:30 p. m.	3:00 p. m.	6:00 p. m.
Controls-Pure culture of N. gonorrhea kept at room temperature	90-100 gonococcus colonies	70 gonococcus colonies	7 gonococcus colonies	3 gonococcus colonies *
Staph. plus N. gonorrhea kept at room temperature	28 gonococcus colonies *	No gonococci	No gonococci	No gonococci
Controls- Pure culture of N. gonorrhea kept at 36° C.	20 gonococcus colonies	4 gonococcus colonies *	No gonococci	No gonococci
Staph. plus N. gonorrhea kept at 36° C.	A few gonococcus colonies *	No gonococci	No gonococci	No gonococci

Symbols: * The longest time that gonococci could remain in broth tubes before being plated out and still show growth upon the plates.

Figure 2



Death Curves of N. gonorrhea at different temperatures with & without Staphylococci.

culture in a broth tube.

(c) Presence of staphylococci at room temperature.

(d) Presence of staphylococci at 36° C.

We can see from Figure 2 that there are differences between the death of the four suspensions of gonococci subjected to as many different conditions. Even standing for one hour at room temperature with a contaminating organism--the suspensions were made at 9:00 a. m. and the first plates were inoculated at 10:00 a. m.--caused the number of N. gonorrhea to appear on the first plates to drop from 90 to 100 to about twenty eight.

Another curve of this graph shows that there is a marked decrease in the number of N. gonorrhea organisms because of standing in a broth culture in the incubator at 36° C., and a still greater decrease when kept at this temperature with the addition of different organisms.

A suspension of a pure gonococcus culture was made in Hartley broth. This was divided into six portions. Three of the broth tubes were left uncontaminated, one being left at room temperature, another at incubator temperature, and the last one was kept in the ice-box. To the other three was added a drop of a suspension of staphylococci. These tubes were kept at the same three temperatures as the controls.

After the suspensions had been standing for six hours at room, incubator, and ice-box temperatures, the plates

made from them showed different numbers of gonococci. The results, shown in Table 6, may be summarized as follows:

(a) No difference was noted in the controls kept at the three different temperatures.

(b) In all cases the gonocccci survived longer when in a pure state than in a contaminated one.

(c) N. gonorrhea colonies alone lasted more than six hours at all three temperatures.

(d) N. gonorrhea colonies plus a contaminant lasted four hours at incubator temperature, four hours at ice-box temperature, and five hours at room temperature.

Although the gonococcus colonies on a plate are less in number when other organisms are present, they will grow extremely close to the contaminating colonies. Examination of many contaminated plates gave no evidence that the gonococci were inhibited to such an extent that they wouldn't grow in the immediate vicinity of the contaminating organisms. This was experimentally proved by first covering a plate evenly with a suspension of gonococci and then streaking it several times with another organism. When this was done using a loopful of Streptococcus viridans, gonocccci pin-point in size were found growing immediately next to the streaks.

Table 6

Inhibition of N. gonorrhea by Contaminants after Standing Six Hours at Ice-Box, Room, and Incubator Temperatures		
Temperature	Controls	N. gonorrhea plus contaminants
Incubator Temperature (36° C.)	Plate covered with gonococcus colonies.	No gonococci
Room Temperature	Plate covered with gonococcus colonies. 0.5 mm. in diameter	No gonococci
Ice-Box Temperature	Plate covered with pin-point gonococcus colonies.	No gonococci

Length of Time Gonococci Will Survive in Original Specimen

Since it may sometimes be impossible to plate a gonorrhea specimen on agar as soon as it is taken, it is important that we know how long the desired organisms will live on the swab. Some experiments were done allowing the specimens to stand at various temperatures, plating them out at intervals. In most cases the swab was pressed against the sides of the tube in which the specimen was collected to remove all the fluid from it and was then discarded. The broth specimen was divided into the number of portions needed for carrying out the experiment.

A specimen taken by Dr. Luten at 10:00 a. m. was plated at 1:00 p. m., 2:00 p. m., 3:00 p. m., and 4:00 p. m. of the same day. Between 1:00 p. m. and 4:00 p. m. the specimen remained at room temperature.

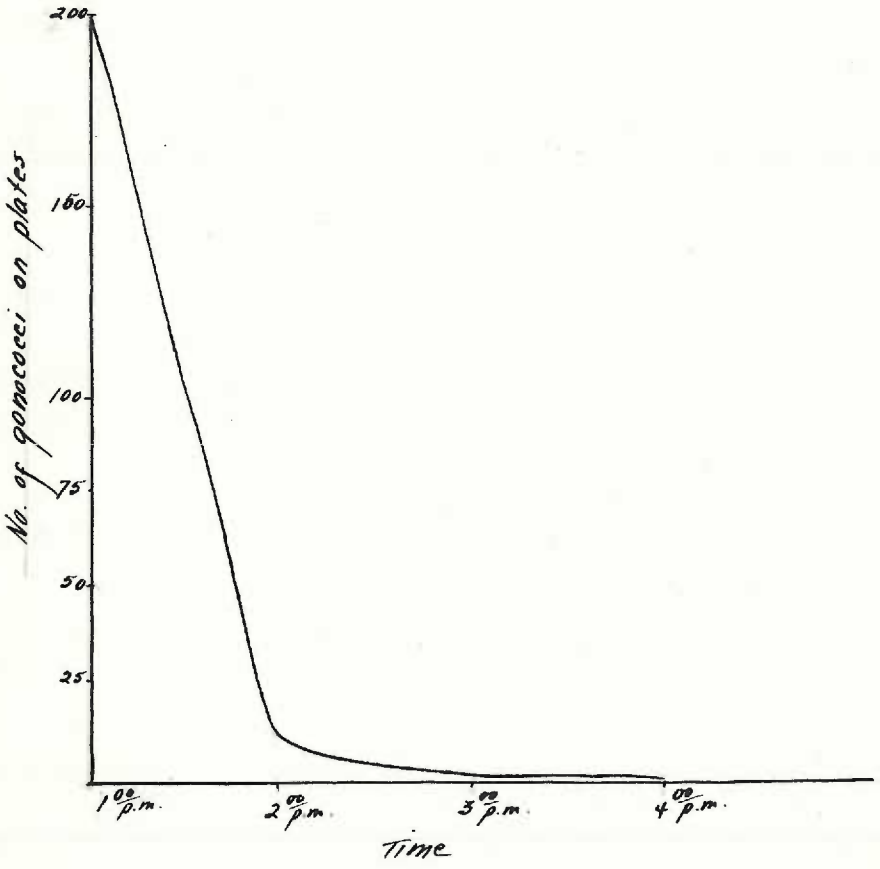
The data on this experiment, which appears in Table 7 and Figure 3, gives evidence pointing to the fact that allowing the specimen to remain several hours at room temperature without being plated has a deleterious effect upon the viability of the gonococcus.

An experiment was run for twenty four hours plating out two like portions of a fresh specimen every two or three hours on Douglas chocolate agar. One specimen was kept at room temperature, while the other remained in the

Table 7

Effects from Standing at Room Temperature Upon the Viability of N. gonorrhea in the Original Specimen				
	1:00 p. m.	2:00 p. m.	3:00 p. m.	4:00 p. m.
Original N. gonorrhea specimen kept at room temperature.	About 200 oxidase positive colonies.	12 gonococcus colonies.	2 gonococcus colonies.	2 gonococcus colonies.

Figure 3



Death Curve of N. gonorrhea in original Specimen at room temperature

Table 8

Effects upon the Viability of N. gonorrhea in the Original Specimen from Standing at Room Temperature and at Ice-Box Temperature		
Time	Specimen kept at room temperature	Specimen kept at ice-box temperature
4:00 p. m.	150 gonococcus colonies.	160 gonococcus colonies.
5:00 p. m.	18 gonococcus colonies.	16 gonococcus colonies.
6:00 p. m.	7 gonococcus colonies.	8 gonococcus colonies.
9:00 p. m.	3 gonococcus colonies.	5 gonococcus colonies.
11:00 p. m.	3 gonococcus colonies.	5 gonococcus colonies.
12:30 a. m.	2 gonococcus colonies.	3 gonococcus colonies.
3:00 a. m.	2 gonococcus colonies.	2 gonococcus colonies.
7:00 a. m.	No gonococci.	No gonococci.

ice-box except when being plated. The contaminating organisms in this case probably exhibited no harmful effects upon the growth of the gonococcus, since the specimen was almost a pure culture.

Table 8 shows that there was no noticeable difference in the viability of the gonococci placed in the ice-box and those left at room temperature. Although the experiment was run for twenty four hours, in both cases the N. gonorrhea organisms were dead in from eleven to twelve hours.

SUMMARY

Although the culture method of diagnosis is superior to the gram-stained smear technic, it is not always reliable. Since N. gonorrhea is a more delicate organism than the other bacteria commonly found associated with it in pathogenic cases, it is entirely possible that the growth of gonococci could be inhibited by the accompanying organisms to such an extent that they would not appear on the plates.

Contaminating organisms were picked from plates made from urethral and cervical specimens from about thirty suspected cases of gonorrhea. After the organisms were isolated in pure culture, they were studied from the point of view of morphology and their cultural and biochemical characteristics. Staphylococci and diptheroids were the most common organisms isolated as contaminants, but streptococci, B. coli, yeast, and B. subtilis were also found associated with the gonococcus.

Inhibition of the growth of N. gonorrhea by foreign organisms can possible be due to either the overcrowding of the plates by the contaminants, the antagonism among the organisms on the plates, or the injurious effects of the metabolic products of the contaminating organisms. Since restraint of the growth of the gonococci is always seen on plates containing other organisms even though there is sufficient room for additional gonococcus colonies,

we know that often there must be a factor involved in causing inhibition of the growth of the gonococcus aside from overcrowding of the plate. When experiments were performed by streaking contaminants across a plate previously inoculated with a pure culture of N. gonorrhea, it was found that gonococci grow immediately next to any contaminant on the plate, provided there is room for them. From this it seems that gonococci will grow in the vicinity of other organisms, although the presence of the latter may cause a reduction in their numbers. Lastly, we know by experimentation that N. gonorrhea is restrained in its growth due to standing in broth with foreign organisms being present.

Pure cultures of isolated contaminants were added to varying dilutions of gonococci. Plates were made from each dilution of the contaminated and uncontaminated tubes. In all cases there was better growth of gonococci on the uncontaminated plates.

Suspensions of pure cultures of isolated contaminants were mixed with pure cultures of N. gonorrhea of the same dilution. The growth of the gonococci was observed by plates which were inoculated from these mixtures which had previously been allowed to stand for various times at different temperatures. Corresponding plates of pure cultures of N. gonorrhea were made to serve as controls. There was noticeable inhibition of the growth of the gono-

cocci, since the organisms grew more profusely on the uncontaminated control plates than they did on the contaminated ones. When the broth mixtures were put in the incubator previous to plating, the inhibition of N. gonorrhea was greater due to the rapid increase of the contaminants.

Original specimens were allowed to stand at various temperatures to determine any harmful effects upon the viability of the gonococci. No marked difference was seen in those kept at room temperature and those kept at ice-box temperature. In both cases no gonococci appeared on the plates twelve hours after the specimen had been taken.

The relationship of the common contaminating organisms to N. gonorrhea has an important bearing upon the diagnosis of gonorrhea by the culture method. Since there are some bacteria that are confusing in that they give a positive oxidase reaction, it is necessary that we know how to distinguish them from the gonococci. It is imperative that we collect a specimen as free from contamination as possible, because there may be only a few gonococci present which could easily be checked in their growth by the foreign organisms so that N. gonorrhea failed to appear upon the plates. Plating a specimen as soon as it has been taken is also essential, if the best possible growth is to be obtained.

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