A STUDY OF METHODS OF PRIMARY ISOLATION CULTIVATION AND IDENTIFICATION OF THE GONOCOCCUS

by

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INTRODUCTION

The inability to cultivate, isolate, and identify the gonococcus with the requisite speed and accuracy necessary in the preliminary diagnosis of gonorrhea, is a problem of considerable importance both to the practicing physician and to the laboratory. So much stress has been placed on the physical and chemical factors of growth, types of media, inoculation methods, and biochemical activities of the gonococcus by various investigators that the initial cultivation and isolation of the organisms has become an undertaking involving considerable understanding and technique on the part of the worker.

Other bacteria, such as the streptococcus, staphylococcus, colon bacillus, and Gram positive diphteroids, comprising the normal flora of the genito-urinary systems in the male and female, tend to make the primary isolation and identification of the gonococcus difficult by ordinary methods.

In this present work we have undertaken to investigate a few of the smaller problems pertaining to the laboratory aspects which have attracted our attention. Owing to the size of present fields of research we have limited ourselves to a study of plate culture methods only.

REVIEW OF LITERATURE

Each phase of the literature having a bearing on the problems related to this work will be listed separately. The work of other investigators will be discussed in detail. Because of the controversial nature of the literature, certain sections will be limited.

Types of media for cultivation, isolation, and identification of the gonococcus. So far as the growth of the gonococcus is concerned, no other culture media has, as yet, superceded the chocolate agar medium of McLeod (1934) in dependability. The introduction of this medium marked the first significant advance in the developement of practical standardized media for cultivation, isolation and identification of the gonococcus. Prior to 1934, numerous media were developed, each more complicated than the other. The results by the investigators who produced them were satisfactory, but, when used by less experienced workers they were inconsistent. Except for further improvement and standardization of certain media by Difco, there has been little or no change in the basic constitution of culture media for the gonococcus.

For routine cultural work, ascitic agar or modifications of blood agar appear to be most generally used. Pelouze and Viteri (1926) developed a brain veal agar which they claimed gave consistent results. Reitzel and Cole in 1938 combined the Difco product, Bacto Brain Heart Infusion with

the brain veal agar, giving a simple medium more adaptable for use in the small laboratory. Price (1937) continued to use the egg albumen medium which he described in 1935, and says it appears to be as simple to produce and as efficient as any of the numerous media with which he has had experience. Gardner (1940) used an alkaline egg white digest with ascitic fluid. Bacto Heart Infusion agar, a modification of the formula of Huntoon (1918), who showed that the more fastidious organisms could be grown on a medium heretofore widely recommended for primary isolation, and still used by many investigators, is the gelatin medium of North (1909). Douglas chocolate agar, and Proteose No. 3 agar, a modification of McLeods' medium, still enjoy the widest popularity for routine cultural work.

The ideal culture medium for the isolation of gonococcus should not only be dependable and easy to prepare, but it should also be one on which the characteristics of the gonococcus can be easily distinguished. For certain types of cultural work, a colorless, transparent medium is desirable, but in this there are certain inherent disadvantages. The medium should be readily adaptable for the oxidase test without too much discoloration. It has been noted, by Leahy and Carpenter (1936) on certain of the clear media, that the oxidase reagent often is absorbed into and discolors the media to the extent that it becomes impossible to distinguish colonies.

The ideal medium is rarely available in the laboratory. Media which must be prepared from complicated formulae before use are undesirable, owing to the complexity of constitution and standardization. One batch may produce one result, while another will yield a different result. It is for this reason the Difco products were used in our work. These preparations are made in large quantities, standardized, and therefore show less variability in ingredients and reaction, and can be stored in the dessicated form for long periods of time without deterioration or change.

Media must also satisfy the organisms' nutritional requirements, and preferable favor the organism being isolated over others that might be present.

Morphology and staining The gonococcus is a Gram negative, non-motile, non-capsulated, oval or spherical diplococcus; 0.8u x 0.6u, with adjacent sides flattened or slightly concave, resembling a pair of kidney beans. Morphologically atypical strains of gonococcus have been described by Cohn (1936), and Göhring (1935). Janet (1936) is of the opinion that in smears correctly stained, the organisms are always Gram negative, while Barbellion (1936) believes that Gram positive forms may occur.

The gonococcus exhibits considerable colony variation. Herrold (1936) described as frequently occuring, a pin-point type which is firmly adherent, with a tendency to grow down

into the medium. Balsamelli (1935) described a more grayish, less transparent, corrugated, dryer appearing form of colony, which is more resistant and exhibits greater vitality than the other forms. In the transition from S-R, the R forms usually retain their normal morphology and staining properties, but a few Gram positive large forms have been reported.

CONDITIONS OF CULTIVATION

Temperature of incubation The optimum temperature of the incubation of the gonococcus is around 36.5 C. Leahy and Carpenter (1936), on the basis of finding that the temperature of the anterior male urethra ranged from 29 C to 31 C when the rectal temperature was 37 C, incubated their primary cultures at temperatures ranging from 29 C to 37 C. The upper range was found to be favorable to growth while the extreme lower range was not. Strains growing at 37 C would not develop at lower temperatures while other strains growing from 33 C to 35 C would not grow at 37 C. Kaplan and Wolfram (1935) incubate their routine cultures at 35 C-36 C.

Initial temperature of medium Winkler (1937), like a number of other workers, thinks it necessary to warm the plates to incubator temperature before making the inoculation. Neumann (1937), whose technique Winkler uses, claims that the plates inoculated at room temperature serve as well.

Consistency of medium The literature, up to the work of McLeod and associates (1934), suggests that the physical consistency or the culture medium may be a more important element than its chemical composition, i.e., that a soft medium is more to the liking of the gonococcus than a stiff dry one, and that failure to recognize this may be responsible for many of the discrepancies between the results of different authors with media in other respects the same.

Caspar (1929) and Siestrop (1930) used a blood water medium with low agar content. Cohn (1932) decided that a dilution of "Levinthal" agar with serum was an advantage and Kadisch and Ruan (1928) found that the lower the agar content(not below 1.3%) in Levinthals' medium, the better were the results obtained in primary isolation of the gonococcus. Torrey and Buckell (1922) recommended a semi-solid medium and Kinsella, Broun, and Garcia (1923), after surveying a large number of media, concurred that a soft agar was best and suggested that the consistency of the medium explained the value of Vedder (1915) starch agar, which is in other respects puzzling.

Since 1934 the advantages of solid media have been so apparent that semi-solid media are seldom used except for sub-culturing and in the maintenance of stock cultures.

Leahy and Carpenter (1936) found that media containing 2% agar had distinct advantages over semi-solid media which frequently failed to adhere to the bottoms of the inverted petri dishes.

Water The question of the proper amount of moisture necessary for the cultivation of the gonococcus is still subject to considerable controversy. It has been noted by Neumann (1936) that in the open incubator, gonococci grow better in stormy weather than in clear. Leahy and Carpenter (1936) found the humidity to be 60% saturation in sealed containers, and 40% to 50% where the containers were not enclosed.

Gaseous tension Although previous investigations had been made into the effect of various gaseous tensions on the growth of the gonococcus, it was for McLeod (1934) and his associates to demonstrate the improved effect of atmospheric air reinforced with CO_2 on the growth of the gonococcus. In a study of the effect of CO_2 on 40 cases he obtained the following results:

TABLE I

Effect of CO_2 on 40 Cases Observed by McLeod

	+ + +	+ +	+	Scanty	50-100	10-50	1-10	0 colonies
air	5	5	3	9	0	1	4	13
8% CO_2 & air	17	11	3	2	1	0	3	3

Leahys' and Carpenters' experience augmented the growing mass of evidence, that, at least with some strains, an increased

CO_2 tension in the surrounding atmosphere exerts a favorable influence on the growth of the gonococcus in primary culture. The following results were obtained after the incubation of 61 strains of gonococci on chocolate agar at 37 C in sealed containers in an atmosphere of 10% CO_2.

TABLE II

Value of Air Reinforced With CO_2

	growth	no growth
air	52	9
air - CO_2	61	0

Neumann (1936), after a careful study, arrived at the same conclusion as Leahy and Carpenter (1936) and McLeod and associates (1934). He further observed that certain strains could only grow in a partial vacuum or increased CO_2 tension, and, after several passages, these strains could be grown under ordinary atmospheric conditions, This conforms to the general experience that CO_2 is unnecessary for maintenance of stock cultures.

Several methods are employed for altering atmospheric tensions. Carpenter and Leahy (1935) pump out part of the air from the containers, replacing it with CO_2 from a tank.

To reduce oxygen pressure, Neumann warms a petri dish and seals it with plastacine. To increase the CO_2 tension, he introduces a fragment of dry ice (CO_2) into the sealed space, which, according to Winkler (1937), brings the tension to about 10%. Spink and Keefer (1937) place a lighted candle in a sealed jar, and allow it to burn itself out; Thompson (1935) describes the addition of calculated amounts of a solution of sulphuric acid to a molar solution of sodium bicarbonate within the jar. Each cubic centimeter of molar sodium bicarbonate solution should furnish 22.4 c.c. of CO_2. With a jar of known capacity the required amount of bicarbonate to furnish the required CO_2 concentration can be calculated.

Accessory substances The exact nature of accessory substances used as media enrichments for cultivation of the gonococcus has not been satisfactorily determined. Balsemelli (1935) says that the gonococcus is easily and quickly isolated in "anti-virus broths" the exact nature of which he did not determine. He believes these broths contain disintegrated bacterial bodies which may produce an appropriate substance for the growth of the gonococcus. Filtrates of certain mold cultures were found, by Sartory and co-workers (1935), to have a certain effect on the growth of the gonococcus when incorporated into a suitable culture medium when blood from different sources was added. Sartory concludes that the blood of patients suffering from gonococcal infections contains

specific, activating, thermolabile, accessory factors for the growth of the gonococcus. They also report that, although normal human and animal bloods produce more abundant and rapid growths of gonococci, other bacteria are also activated at the same time, so that these bloods are of no advantage in culturing from mixed infections. This has been generally disproven by the development of the Difco Proteose No. 3 agar. McLeod (1934) used whole blood as a means of supplying accessory factors. Ascitic fluid added to veal infusion agar was used by Cohn (1936) and other workers with considerable success. The main advantage in using ascitic fluid lies in the fact that it contains no formed elements which would tend to discolor the otherwise clear culture medium. Peizer (1939) employed horse plasma and hemoglobin enrichments, while Cohn and Kruger (1940) made use of hemolyzed beef or sheep blood in media for isolation of the gonococcus.

Dyes Though a few scattered observations had been made at various times on the effects of aniline dyes on bacteria, it was for Churchman (1912) to first investigate them thoroughly. He found that on media containing gentian violet in high dilutions, 90% of the Gram positive organisms were killed, while the remaining 10%, the acid fast organisms in particular, were not affected. Similarly, though 90% of the Gram negative organisms were resistant, 10% were susceptible to the action of the dyes. This difference in resistance between Gram

positive and Gram negative organisms is not sharply defined. There is also considerable variation in the susceptibility of different species of Gram positive organisms. Garrod (1933a) has shown, for example, that staphylococci are much less resistant to the violet dyes--crystal violet, methylviolet, Hoffman violet, gentian violet, Dahlia--than streptococci. Garrod (1933b) has further found that aniline dyes in general are more bacteriostatic for Gram positive than Gram negative organisms.

It was not until the work of Gardner (1940) that an aniline dye was included in a medium for the isolation of the gonococcus. To a modified egg white digest medium he added Nile Blue A (National Aniline Company) and through its inhibitory action, succeeded in reducing the growth of organisms other than gonococci by at least 50%. In the dilution used there was no inhibitory effect on the gonococci and when other bacteria grew, it was noted that the former organism grew more rapidly and absorbed more of the Nile Blue A than the other bacteria present.

No mention has been made by Gardner, or elsewhere in the literature, of the effects of other aniline dyes--crystal violet, basic fuchsin--on bacteria in the presence of the gonococcus.

Carbon Glass and Kennet (1939) report a series of experiments using the following forms of finely divided carbon in a phosphate buffer solution and a measured amount of liquid nutrient agar:

Commercial Blood Charcoal, Graphite, Medicinal Charcoal (Merck) and in addition to these chalk, kaolin, and red oxide of iron.

The carbon phosphate buffer mixture and liquid nutrient agar was poured into plates, allowed to harden and inoculated both with pure strains of gonococci and pus containing this organism. Blood charcoal, in dilutions ranging from 1:200 to 1:400, was found to give the greatest growth enhancement.

The following chart, including cases diagnosed by this method, will convey an idea as to the efficiency of the charcoal plates.

TABLE III

The Isolation of Gonococci on Charcoal Agar from Pus.
(Data from Glass and Kennet)

Case No.	Treated	g.c. in smear	Number of colonies appearing on				
			blood agar		charcoal agar		agar
			24 hrs.	48 hrs.	24 hrs.	48 hrs.	48hrs.
487	No	+	Inn.	Inn.	Inn.	Inn.	0
503	Yes	+	"	"	"	"	0
557	No	+	"	"	"	"	0
733	No	+	55	55	45	45	0
785	No	+	Inn.	Inn.	Inn.	Inn.	50
797	No	-	200	200	200	200	40

Charcoal plates contain 1:200 or 1:400 commercial blood charcoal (CBC)
Inn. Innumerable, i.e., over 250 colonies

The charcoal plates contain 1:400 or 1:200 CBC
Inn. Innumerable, i.e., over 250 colonies
exerpt from chart containing 25 cases

Growth on the charcoal plates took longer (up to 48 hours) before a count could be made, but the results were always comparable to the blood agar plates.

Glass and Kennet found that through the addition of certain preparations of carbon to nutrient agar the suitability of the medium was greatly increased for the growth of the N. gonorrhoeae and N. intracellularis and, in the case of the former, only is particularly evident when incubation is carried out in 1.5 to 5% CO_2. The effect produced, they concluded, was by the blood charcoal, heated sugar charcoal, and graphite, but not by the commercial sugar charcoal or Mercks' medicinal charcoal. The effect was not due to soluble material or matter decomposable at 1000 C in one hour. To enhance the growth, the carbon particles must be present in the medium during incubation. Mere exposure to the carbon fails to produce results.

Warburg (1913-14) has shown that certain charcoals catalytically oxidize oxalic acid to CO_2 and water in the presence of oxygen. Warburg and Negelein (1921) and Warburg (1921) showed that cystine, tyrosine, and leucine are similarly acted upon. He contended that charcoals containing nitrogen and iron were more active than those that did not. Glass and Kennet arrived at the same conclusion.

Oxidase reaction Since 1937 the oxidase reaction as a means of identification of the gonococcus colony has attracted considerable attention. Many workers who are accustomed to recognizing the colonies by inspection alone have been prone

to discount this test as a means of identification, but after trying it, not a few have changed their minds. There are several compounds belonging to the p-phenylene diamine group, which give this reaction to some degree, but, after using all of the members of this dye family in the tests, Gordon, McLeod, and Ellingsworth (1929) came to the conclusion that tetramethyl paraphenylene diamine hydrochloride was the most desirable, in that it did not exhibit the same degree of toxicity to the gonococcus as the other members of this group. L. Thompson (1936) and Spohr and Landy (1936) prefer the tetramethyl compound. Leahy and Carpenter (1936), who tried both the dimethyl and tetramethyl compounds, prefer the dimethyl, deciding that the tetramethyl compound was inferior because it discolored the medium, to a greater degree than the dimethyl.

In L. Thompsons' (1936) report on the specificity of the dyes for gonococcus, as well as in the work of Gordon and McLeod (1926), (verifying the results of Schultze (1910) and Kramer (1910)), there is ample evidence that the gonococcus is not alone in its oxidase producing ability. In a period of 8 months, Thompson found out of 1,100 cultures, 11 instance in which there were confusing positive oxidase tests.

Leahy and Carpenter (1936) compare inspection with the oxidase test as a means of recognizing the gonococcus colonies in cultures of 146 strains. Both were positive in 86% of the cases, and the oxidase picked up 21 of 14% of those missed by inspection giving a 15% superiority for the oxidase method.

TABLE IV

Advantage of the Oxidase Test Over Direct Inspection
In the Identification of 146 Strains of the Gonococcus
(After Leahy and Carpenter)

	Strains Identified	Percentage Positive
Inspection positive, oxidase test positive	125	86%
Inspection negative, oxidase test positive	21	14%

Schultze and Kramer (1910), and Gordon and McLeod (1928) subjected various organisms to tests with dimethyl p-phenylene diamine and a-napthol and found that those organisms having the enzyme catalase were capable of entering into a reaction with these compounds. Bacteria entering into this reaction had not been previously recognized as peroxide formers. Because of their catalase, or some other factor, the peroxide they produce does not diffuse into the surrounding medium. On the other hand, bacteria, from which the peroxide diffuses into the surrounding medium, are apparently devoid of peroxidase and give no reaction unless peroxide is supplied. Only those organisms growing in the presence of oxygen are capable of entering into reactions of this nature.

Gordon, McLeod and Ellingsworth (1929) found the para

compound, as well as the mono and dimethyl derivatives, toxic to the colonies of organisms treated, with killing times ranging from 2 to 15 minutes. This was undesirable, making it impossible to transplant colonies. This lead to an investigation of related compounds, resulting in the use of the tetramethyl compound which was found to be only slightly toxic, the organisms remaining viable for long periods of time after application of the dye.

The mechanism within the organisms is apparently due to the action of the enzyme catalase on the peroxide formed within the cell body as the result of the oxidation of lactic to pyruvic acid by the a-hydroxyoxidase system, (Barron and Hastings)(1933 and 1936), of the gonococcus and the subsequent release of oxygen. So far, this is the only oxidation-reduction system that has been investigated in the gonococcus.

The a-hydroxyoxidase system activates lactic acid* producing a rearrangement of its' electronic structure so that it becomes electronically active. In this system there are two active components, the a-hydroxyoxidase and a thermolabile ferric hemin-like compound which is destroyed by heating at 50 C for two hours.

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* The following oxidation-reduction system is proposed for the gonococcus.

(ON NEXT PAGE)

TABLE VI

Effects, Color Changes, and Relative Toxicity

of the Para-phenylene Derivatives

Derivatives	Color Changes with H_2O_2	Effect of Oxidase Active Bacteria Exposed to Reagent and Air	Toxicity
para-phenylene diamine HCl	---	slow formation of black pigment	Very toxic
mono-methyl para-phenylene diamine HCl	faint, yellowish brown	rapid formation of black pigment	very toxic
di-methyl para-phenylene diamine HCl	maroon(Würster's Red) to black	slow formation of black color after preliminary maroon stage	toxic within 2-5 min.
tetra-methyl para-phenylene diamine HCl	purple (Würster's Blue) to deep purple	formation of deep purple color after preliminary purple stage	non-toxic under 30 min.

(1) $2HCH_3\text{-}\underset{\underset{HO}{|}}{CH}\text{-}COOH$ (Lactic acid) —activating enzyme of a-hydroxyoxidase→ $2\ CH_3\text{-}\underset{\underset{HO}{|}}{C}\text{-}COOH + 2H$ (activated lactic acid)

(2) $2\ CH_3\text{-}\underset{\underset{HO}{|}}{C}\text{-}COOH + 2Fe^{+++}$ hemin —thermolabile component of a-hydroxyoxidase system→ $2\ CH_3\text{-}\underset{\underset{O}{|}}{C}\text{-}COOH + 4H$

(3) $4\ Fe^{++}$ hemin $+ 2O_2 \longrightarrow 4\ Fe^{+++}$ hemin $+ 4O$

(4) $4H + 4O \rightarrow 2\ H_2O_2$

(5) $2H_2O_2$ —catalase→ $2H_2O + O_2$

(6) O_2 + Tetramethyl p-phenylene diamine HCL (Wurster's Blue) End product of oxidation of dye

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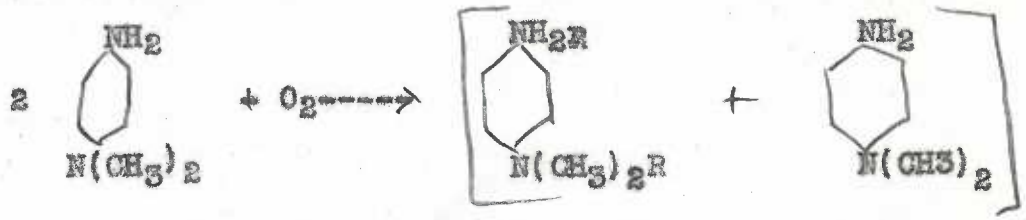
In the presence of peroxide, the paraphenylenediamine compound yields no color change; the monomethyl derivative produces a yellow brownish coloration; the dimethyl compound, a black color after a preliminary maroon stage. The maroon color complex is known as Wursters' red (1879) and the blud color produced through the interaction of the tetramethyl compound is Wursters' blue (1879).

Figure 1

(1)

Di-methyl p-phenylene
diamine hydrochloride



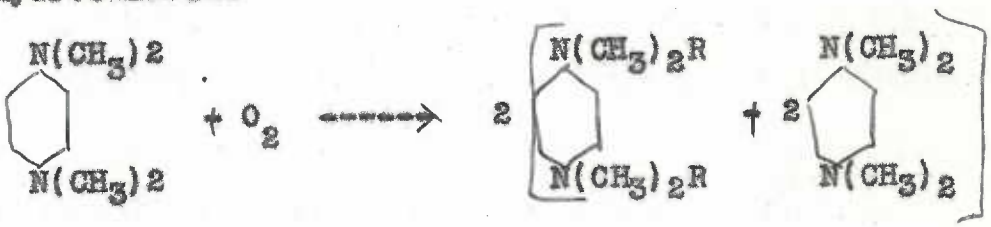
Wurster's Red (1879)

Dye complex result of interaction between oxygen and di-methyl p-phenylene diamine hydrochloride.

Figure 2

(2)

Tetra-methyl p-phenylene
diamine hydrochloride



Wurster's Blue (1879)

Result of interaction between oxygen and tetra-methyl p-phenylene diamine hydrochloride

Superiority of cultural over smear methods of diagnosis

Interest in the superiority of the cultural method for diagnosis of gonorrhea has been stimulated by repeated demonstrations of inaccuracies arising from the microscopic method. From the diagnostic standpoint the culture of the gonococcus is extremely important because of the fallacies of the Gram reaction and the

us of sulfanilamide in the present day treatment of gonococcal infections.

Single smears stained by Grams' method were accepted as being sufficient by Herrold (1931) in the presence of acute clinical symptoms and findings. Using a modified McLeods' method (1934) for the diagnosis of gonorrhea in the adult female, Carpenter, Leahy and Wilson (1938) reported the gonococcus in 205, or 92%, of 223 positive specimens submitted for examination. Only 107, or 48%, of the specimens showed the gonococcus in smears. The cultural method was 191% superior to the smear method.

TABLE VII

Superiority of Cultures over Smears
(From Carpenter, Leahy, and Wilson)

Positive Specimens	Smears Positive	Cultures Positive	Superiority of Cultures over Smears
223	48%	92%	191%

TABLE VIII

Superiority of Culture Over Smear Methods of Diagnosis; A Summary of the Results of 3100 Routine Examinations from Several Investigators*.

Cultures Examined	Smears Positive	Cultures Positive	Superiority of Cultures Over Smears
(1) 2062	7.75%	14.5%	187%
(2) 195	7.7%	17.5%	227%
(3) 843	12.7%	24.5%	191%
Totals and 3100 Averages	9.38%	18.5%	195%

Explantion of signs:
(1) McLeod & Co-workers (1934)*
(2) Spohr & Landy (1936)*
(3) Carpenter & Leahy (1937)*

INTRODUCTION
TO
EXPERIMENTAL WORK

General objectives Since no evaluation of culture media has appeared in the literature since the work of Mak (1936), we have undertaken a survey of plating media used in the diagnosis of gonorrhea. In this study will be included methods of isolation, culture media, and accessory substances employed in routine cultural work. Further observations were made on the inhibitory action of the dye, Nile Blue A (Gardner(1940)), as well as a study of the effects of two more aniline dyes on the Gram positive organisms frequently associated with the gonococcus during primary isolation.

The use of blood charcoal as an accessory substance for the growth of the gonococcus came to our attention after reviewing the work of Glass and Kennet (1939), and portions of their work was repeated with modifications. Though it is generally conceded that a moist surface is necessary for the growth of the gonococcus, the effects of dry, moisture-free surfaces on the growth and colony size of the organisms were studied with results proving quite interesting in the light of commonly held views.

Though we have not contributed any one thing of far-reaching significance in the laboratory diagnosis of gonorrhea,

we feel that through repetition and substantiation of previous work, and the additional ideas gained through our own observations, we may, in a small way, add to the already fast accumulating knowledge on combating this disease.

METHODS AND MATERIALS

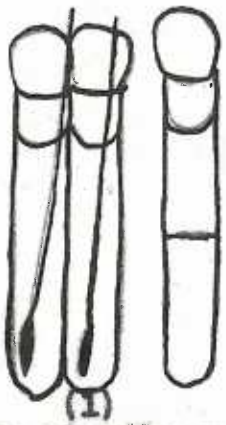
Although little is said in the literature on inoculation methods, we feel that much is assumed by many investigators in regards to the proper execution of this procedure as an adjunct to the diagnosis of gonorrhea.

Methods of inoculation The inoculation methods employed by laboratory workers for the primary isolation of the gonococcus vary widely. Some use the original swab upon which the specimen was taken, using it to streak their plates; others use sterile transfer pipettes and glass dollies for spreading the inoculum uniformly over the surface of the medium. The platinum loop can be used, but, with it, it is difficult to transfer material to the medium in quantities.

Of the methods mentioned, transfer pipettes and glass dollies were found to be most satisfactory. Rubber bulbs attached to the pipettes made it possible to avoid accidental contamination of the mouth and allowed for greater accuracy in transfers and dilutions.

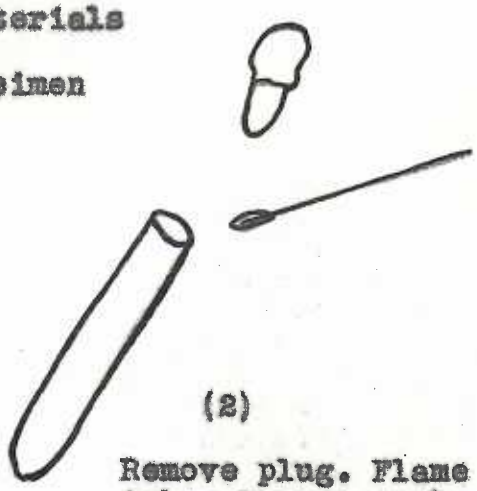
All specimens were obtained on sterile swabs which were immersed in 1c.c. of a sterile solution of 1% peptone water or broth. This insured adequate moisture for the organisms till they could be transferred to culture plates. The use of the dollies tended to give more uniform inoculated areas and prevented drainage of material from the surface of the media onto the cover of the petri dish.

Methods and Materials for Obtaining Specimen



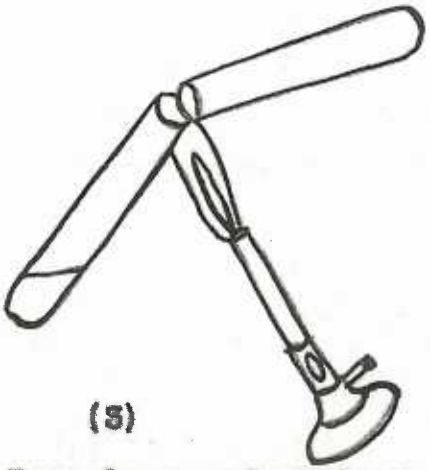
(1)

Have sterile swabs and a 1% solution of peptone water available in test tubes



(2)

Remove plug. Flame tube. Remove swab and take specimen.



(3)

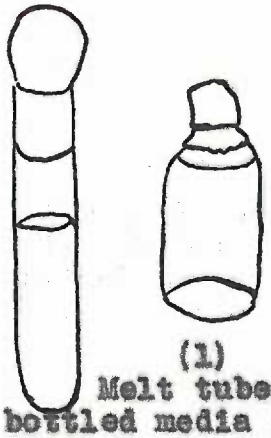
Pour 1c.c. of peptone water into tube.

(4)

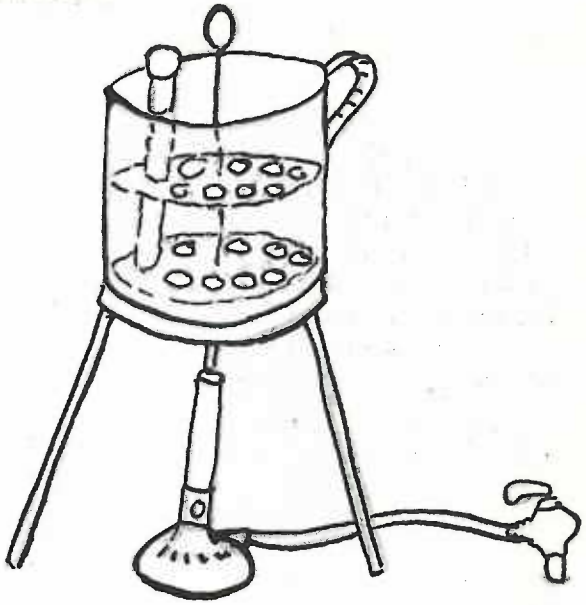
Return swab to tube, immersing well in broth. Either transfer specimen to plate or store in refrigerator.

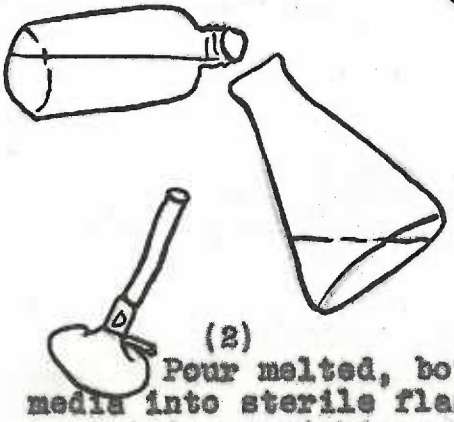
Figure 3

Methods and Materials
for
Pouring Plates

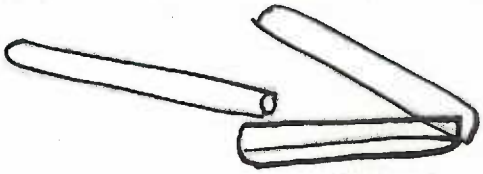


(1)
Melt tubed or bottled media in water bath.

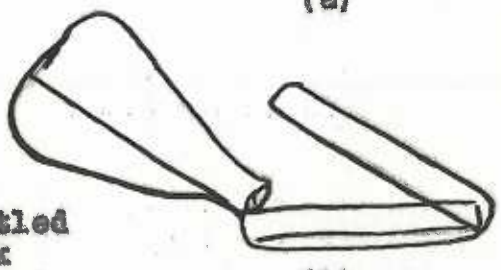




(2)
Pour melted, bottled media into sterile flask containing enriching substance and mix by rotation.



(a)



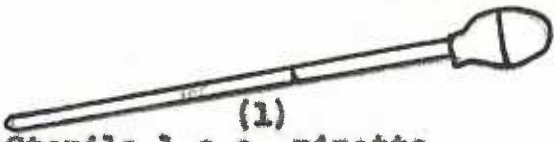
(b)

(3 a,b)
Pour media into petri dish with frequent flaming of mouth of flask.

(4)
Allow medium to harden.

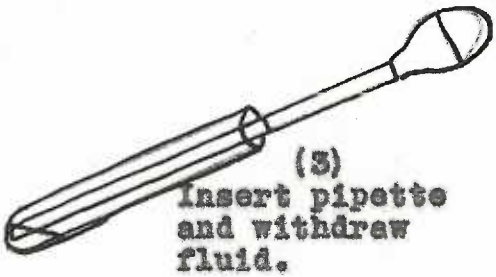
Figure 4.

Methods and Materials
for
Inoculation

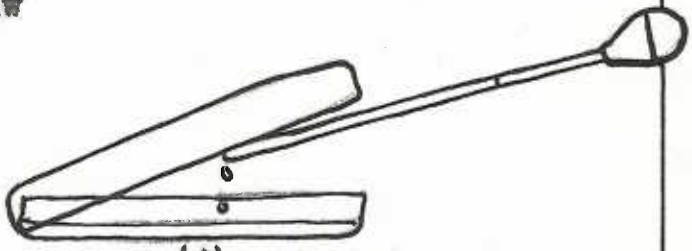


(1)
Sterile 1 c.c. pipette with rubber bulb attached.

(2)
Press swabs against sides of tube working material into broth. Discard swabs.

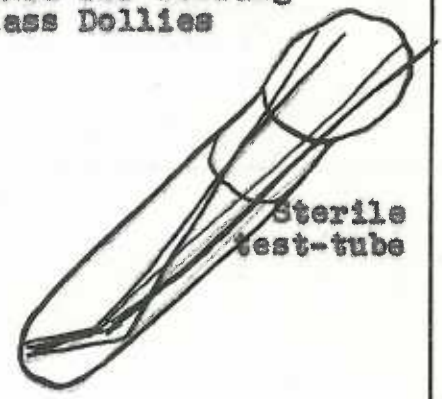


(3)
Insert pipette and withdraw fluid.



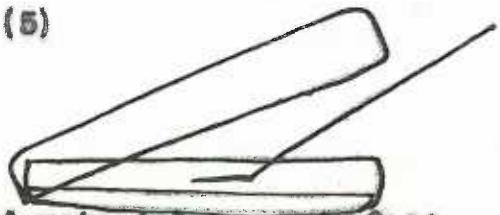
(4)
Inoculate plate.

Method for Storing
Glass Dollies



Sterile test-tube

(5)



Spread material over surface of medium with glass folly.

Figure 5

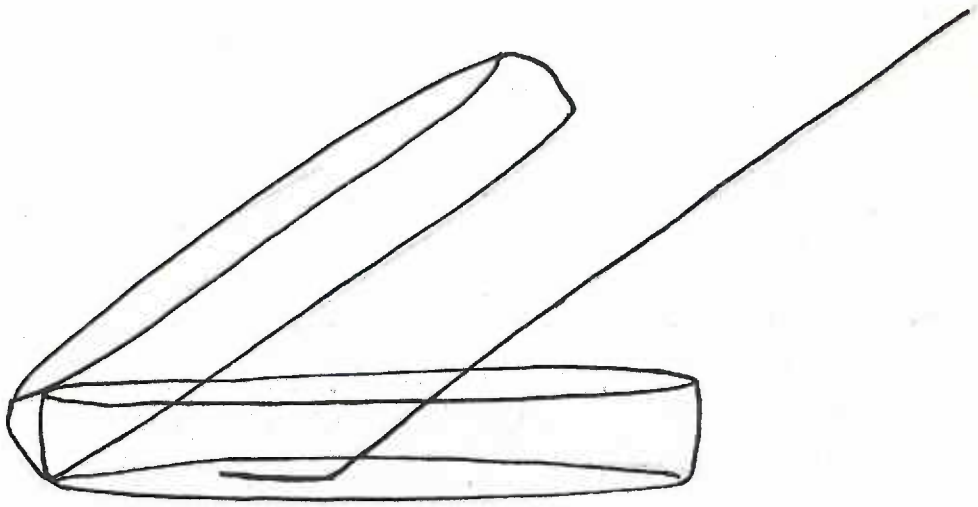


Figure .

Technique of Spreading Inoculum
With Glass Dolly

Raise cover of petri dish in manner indicated in above figure. Rest dolly on surface of medium. Spread inoculum with a cross-wise and circular motion till all material is worked well into the medium. Dolly should be handled in such a way that no marks are left on the smooth surface of the plate.

Figure 6

Temperature of medium for inoculation As pointed out in the review of the literature, many investigators stress the warming of plates to incubator temperature before inoculation. So far no detectable difference in the growth of the gonococcus on either heated or unheated plates has been observed. The gonococcus grew profusely on plates inoculated at room or refrigerator temperature. Since the gonococcus may be stored at refrigerator temperatures for several hours without any noticeable effect on its viability and the temperature of the plates is not a contributing factor to its survival, we feel that there is no necessity for warming either plates or cultures before transfer.

Temperature of incubation Our best results have been obtained at a temperature of 36.5 C, although there were occasional strains which failed to grow at this temperature. An incubator should be used which is not subject to wide temperature variations, since changes of 2 C or 3 C either side of the optimum temperature will often kill a new strain during the initial 48 hour incubation period.

Control of moisture and humidity during incubation In freshly poured plates, there is an adequate supply of moisture present in the medium to maintain the proper humidity when they are incubated within a closed container. Should the plates be quite dry, 5 or 10 c.c. of water may be poured into the jar before incubation, or a folded, dampened, paper towel can

be placed in the bottom of the jar.

When separate petri dishes are to be incubated, wide rubber bands, after the method of Petroff and Steenken (1930), can be placed around the edges of the petri dishes in such a manner as to seal the space between the bottom and cover, thus maintaining atmospheric conditions suitable for growth.

Carbon Dioxide Carbon dioxide was obtained in cylinders and introduced into the jars following a method modified after Leahy and Carpenter (1936). All cultures, except those so designated, were grown in atmospheres reinforced with CO_2. We are of the opinion that practically all strains of the gonococcus are carbondioxidophillic, at least during primary isolation.

Our apparatus for handling the CO_2 is simple. We found it easier and faster to operate than Leahys' and Carpenters'.

Our apparatus is composed of a glass bottle, with a tubulature near the bottom, an ordinary 2 liter bottle filled with water and calibrated in cubic centimeters, a glass stopcock, and a cylinder of CO_2 . The glass tubing from the elevated bottle extends nearly to the bottom of the calibrated flask, passing through a two holed stopper containing the glass stop-cock to which a rubber tube is connected to the CO_2 tank and the stop-cock opened. As the gas pressure increases in the bottle, the water is displaced into the upper bottle. Upon obtaining the desired amount of CO_2, the stop-cock is closed. The rubber tubing is detached from the cylinder and placed in the incubation

EXPLANATION

(A) 5 L. glass bottle with tubulature near bottom.

(B) 2 L. glass bottle such as is ordinarily used for acids and bases.

(C) Glass stop-cock.

(D) 5 lb. glass coffee jar with screw top for incubation of plates.

(E) Carbon dioxide in cylinder equipped with high pressure valve.

(F) Rubber tubing.

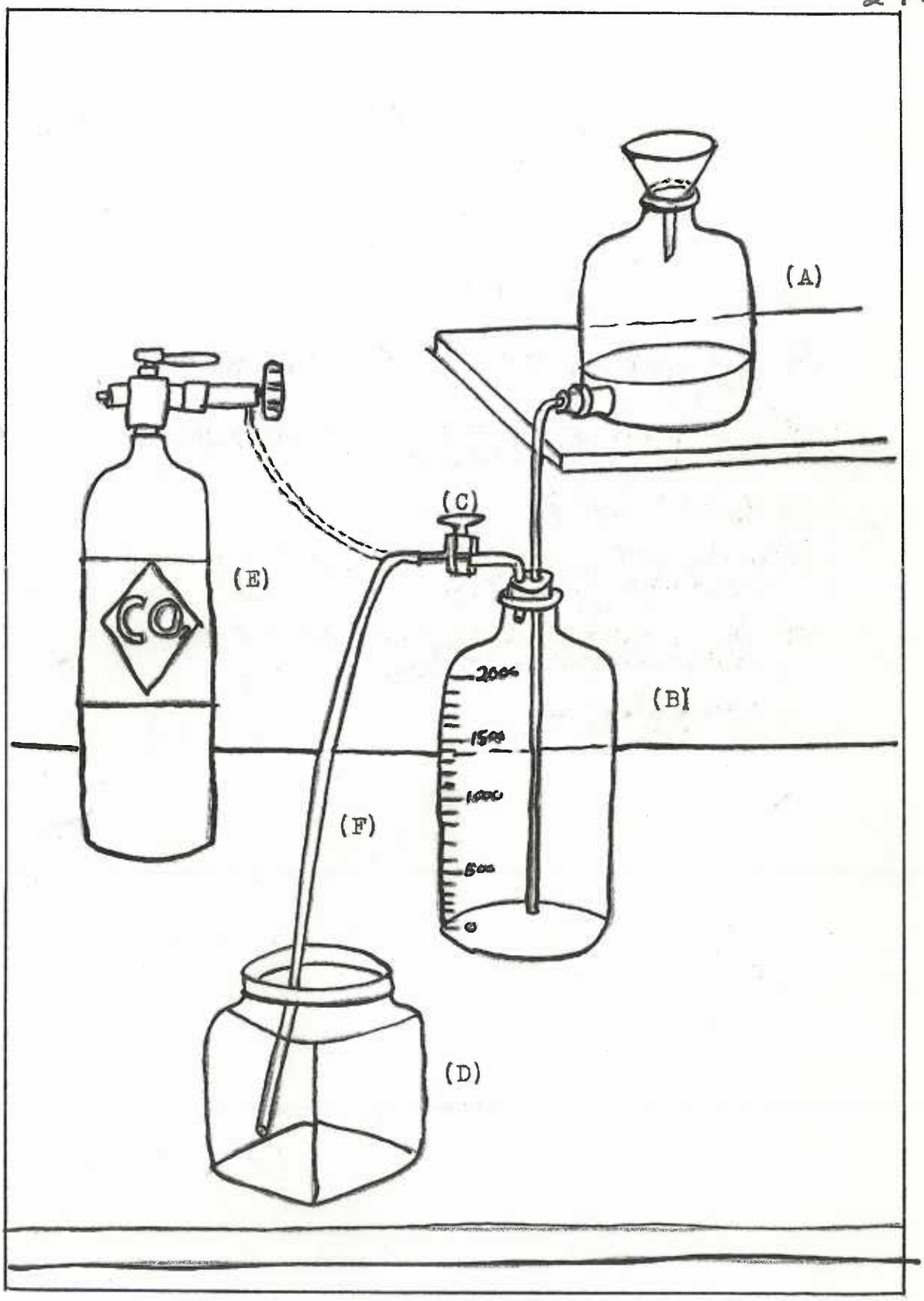


Figure 7

jar. The weight of the displaced water forces the CO_2 into the jar. When the capacity of the incubation jar is known, there is little difficulty in computing the proper amount of gas to be used.

Carbon dioxide, being heavier than air, forms a layer on the bottom and the loss of gas from the jar is practically nil. The lid is placed on the jar after the tube has been removed.

Preparation and storage of media Whenever possible, the dessicated Difco products were used. These were chosen because they were well standardized, consistent, and reliable in reaction. Such media as could not be obtained through Difco were prepared in our own laboratory.

Directions for preparation of the dessicated media were obtained either from the package or the Difco manual. Anyone interested in seeking more specific information on methods is referred to the Difco Manual.

When it was necessary to prepare and store media, test tubes or 100 c.c. screw cap bottles were used.

Storage of accessory substances Bloods and ascitic fluid were stored in the refrigerator in sterile flasks. Bacto-Hemoglobin was tubed in 10 c.c. quantities and stored till needed in the refrigerator.

Summary of media used The plating media used in our work were Bacto Proteose No. 3 agar, Bacto Brain Veal agar, Bacto North Gelatine agar, Bacto Heart Infusion agar, and Douglas agar. Brain veal agar, made after the formula of Peizer (1940), is used in one instance but no thorough study of it was made. (See table)

Bacto Proteose No. 3 agar This media is composed of proteose peptone, dextrose, sodium chloride, disodium phosphate, and agar. The resulting media is one which permits the growth of the gonococcus not only in pure culture, but also allows its development from the mixed flora encountered in chronic gonorrheal infections.

The development of this medium was premised on the stipulation that it possess a high degree of efficiency, lend itself readily to practical use, be of simple composition, and easily duplicatable. Drs. Carpenter, McLeod, and Herrold, who aided in the development of this medium, feel that it is superior to Bacto North Gelatin agar or Bacto Brain Veal agar for use in the isolation of the gonococcus.

Bacto Brain Veal agar Bacto Brain Veal agar is composed of extractives of fresh calf brain and veal in addition to proteose peptone, sodium dihydrogen phosphate, sodium chloride and agar. This medium was devised by Pelouze and Viteri (1926) following a study of the cultural requirements of the gonococcus. Garcia (1930) was well satisfied with this medium and found that it

fulfilled the growth requirements of the organisms. Saccone (1936) used Bacto Brain Veal agar for preliminary isolations. Reitzel and Kohl (1938), by combining Bacto Brain Heart Infusion agar with Bacto Brain Veal agar, made a simple medium which was claimed by them to be more adaptable for use in the small laboratory than Bacto Brain Veal agar alone.

Bacto Heart Infusion agar Bacto Heart Infusion agar is a solid medium prepared with extractives of fresh meat, proteose peptone, sodium chloride, and agar. Huntoon (1918) was one among many investigators to show that organisms of a highly parasitic nature could be grown upon such a medium without enrichment. Proteose peptone was found to be better suited to the nutritional requirements of pathogenic bacteria than the Bacto Peptone originally used by Huntoon in the preparation of his "Hormone" medium.

Bacto North Gelatine agar Bacto North Gelatine agar is a modification of Norths' medium (1909) recommended for use in the cultivation of the gonococcus and meningococcus. It is more complex in ingredients than any medium heretofore mentioned, being composed of veal infusion, proteose peptone, gelatine, soluble starch, isoelectric casein, sodium chloride, and agar. Spohr and Landy (1936), and Beck (1938), reported that Bacto North Gelatine agar was an excellent medium for the cultivation of the gonococcus following the addition of whole blood.

Douglas Agar Douglas agar is prepared by the addition of agar to Hartleys' Broth, which is composed of a meat beef extract, sodium carbonate, and Cole and Onslows' pancreatic extract with chloroform. As reported by McLeod (1934), this makes an excellent medium for the gonococcus, but its greatest fault lies in the difficulties of its preparation.

EXPERIMENTAL WORK

Effects of the drying of the medium on growth and colony size

It has been observed that the gonococcus grows best in the presence of an adequate supply of moisture, either on the surface of the medium or in the atmosphere coming in contact with the medium. This is, generally speaking, true, but we have had occassion to wonder what the effects on the growth and size of the colony would be when grown on plates having pre-dried surfaces. Douglas chocolate agar plates were stored under a bell jar for a period of 43 days, inoculations being made at frequent intervals with both pure, freshly isolated strains, and mixed specimens. The bell jar covering the plates was raised from the surface of the table by means of 1" pieces of wood to insure circulation of air and enable moisture to escape. During the 43 day period in which the plates were under the jar, there was no evidence of moisture on the plates. The surfaces of the media did not get dry enough to crack or become discolored.

All inoculations were made from suspensions of the gonococcus in 1% proteose peptone solutions and inoculated in amounts varying from 0.1 to 0.3 c.c. by means of 1 c.c. graduated transfer pipettes. The inoculum was spread over the surface of the medium with glass dollies, taking care to work it well into the surface. A more uniform growth of organisms could be obtained in this manner than by streaking the plate directly with the swab.

The sizes of the colonies grown on these plates varied from pin-point to 1.5 mm. in diameter. The following table shows the relative colony sizes on plates inoculated at varying intervals over a period of 43 days. As can readily be seen, the variation in size after the 13th day to the 43rd day was not noticeable. The largest colony at any one time, even on the freshly poured plates, was 1.5 mm.

TABLE IX

The Effects of Drying on Colony Size of the Gonococcus Plated on Douglas Agar

	Days					
	2	4	7	10	13	43
Colony size in millimeters	0.5-1.5mm	pin point to 1mm	0.5-1.5mm	pin point to 0.5mm	pin point to 1mm	pin point to 1mm

All of the plates were incubated in a 10% CO_2 tension at 36.5 C for 48 hours. The colonies reacted with the oxidase reagent (tetramethyl paraphenylene diamine hydrochloride). Discoloration of the medium was not too pronounced. At the end of the incubation period, the colonies exhibited smooth glistening surfaces and edges. No appreciable difference could be noted

between the colony sizes of the gonococcus grown on dried plates and those grown on the freshly poured plates.

There are disadvantages in keeping plates on hand for extended periods of time. Contamination from airborne bacteria and molds is a constant risk.

Effects of pre-incubation at 36.5 C on Douglas Chocolate agar plates

Douglas Chocolate agar pour plates were placed in an incubator for three days at 36.5 C and then inoculated with a suspension of the gonococcus in pure culture. The plates were then incubated in a 10% CO_2 tension for 48 hours. From all indications, the period of pre-incubation has very little effect, if any, on the growth of the gonococcus. The size of colonies is apparently a factor of the dilution and the prescence of other organisms in the inoculum, as well as being due to the presence of moisture in the plates or jar.

EVALUATION OF CULTURE MEDIA

Since several media and accessory substances were used in the following comparisons, the experimental work will be presented in chart form wherever possible. The culture and inoculation methods used were described and illustrated in a previous section.

By performing a series of experiment in which varying dilutions of cultures were used, it was found that in almost every instance, where growth conditions were favorable, the conococcus colonies on plates were larger in the higher than in the lower dilutions. This is probably due to the ease with which the products of metabolism can diffuse and be handled through the buffering action of the media. Colonies close together, tended to be smaller, varying from pin-point in size to 0.5 mm. in diameter. Well separated colonies were larger, ranging from 0.5 mm. to 3 mm. in size. Colohies as large as 6 or 8 mm. in diameter have been observed, but in routine work the largest encountered was approximately 3mm.

The following table is intended to show the relation of colonies encountered on three different media, using different enrichments. (The Peizers' Veal medium with horse plasma-hemoglobin enrichment was not one of the media subjected to comparative tests. For fuller discussion of the Peizer medium see Peizer, 1939). These media give fairly consistent results. Proteose No. 3 Bacto Hemoglobin and Douglas Chocolate agar,

TABLE X

Effect of Varied Media and Enrichments on the Colony Size of the Gonococcus Using Pure Cultures in Different Dilutions.

Medium	Dilutions			
	1:1	1:10	1:100	1:1000
Protease No. 3 Bacto Hemoglobin agar	inn. 0.5-2.5 mm.	150-200 0.5-2.5 mm.	30 0.5-3mm.	9 0.5-3mm.
Douglas Chocolate (horse blood) agar	inn. pin-point to 1mm.	45 pin-point to 2mm.	100 pin-point to 3mm.	15 0.5-2.5 mm.
Peizer Veal Horse-Hemoglobin Plasma agar	inn. pin-point to 1.5 mm.	50 pin-point to 2mm.	65 pin-point to 2.5 mm.	22 pin-point to 2.5 mm.
Peizer Veal Horse-Hemoglobin agar	inn. pin-point to 1.5 mm.	150 pin-point to 2mm.	24 pin-point to 1.5 mm.	20 pin-point to 1.5 mm.

Figures indicate number of colonies per plate and relative diameters of colonies

inn. Innumerable (over200)

being the routine standard media, are very close together in their ability to support growth. Cultures 48 hours old were first inoculated in 1 c.c. 1% proteose peptone and diluted from 1:1 to 1:1000 before being transferred to the culture plate. In the lowest dilution all plates were so heavily covered with colonies that accurate counts were impossible. It is of interest to note, that the 1:1 dilution on Proteose No. 3 Bacto-Hemoglobin agar gave colonies almost twice as large as the other plates in which different accessories and dilutions were used. Colonies on the plates in the higher dilution series grew up to 2.5 mm. in diameter, showing little or no difference in the ease with which the media supported growth.

Of all the media used, with the exception of Douglas Chocolate agar, Proteose Bacto-Hemoglobin No. 3 agar gave the best results for both primary isolation and secondary cultivation. The colonies usually were large and wellseparated, and about 3 mm. in diameter. Douglas Chocolate agar, though giving excellent and comparable growth, can be discarded in favor of Proteose No. 3 Bacto Hemoglobin. This is because Hartleys' Broth is difficult to prepare and keep on hand in the small laboratory.

Ascitic fluid can be used in media in varying quantities when no other means of enrichment are available. Since it has no formed elements, clear media can be obtained, but, in our hands, this enrichment did not yield consistent results and the media stained readily with the oxidase reagent. Colonies grew

Table XI

Growth and Colony Sizes of the Gonococcus Using Different Media and Enriching Substances

Medium and Enrichment	Colony Size
Proteose No. 3 agar (D.S.) with Bacto Hemoglobin	0.5-2.5mm.
Proteose No. 3 agar (D.S.) 10c.c. ascites/10cc. medium	0.2-1 mm.
Proteose No. 3 agar (S.S.) 2 c.c. Bacto Hemoglobin/plate	2.0-2.5mm.
Proteose No. 3 agar (S.S.) 2 c.c. ascites/plate	Pin-point to 1.5mm.
Douglas Chocolate agar 2 c.c. horse blood/ plate	Pin-point to 1.5mm.
Douglas agar 2c.c. ascites/ plate	---------
Douglas agar 2 c.c. Bacto Hemoglobin/plate	Pin-point to 0.1mm.
Peizer Veal agar * horse hemoglobin-serum	Pin-point to 1.5mm.
Peizer Veal Agar * horse hemoglobin	Pin-point to 1.5mm.

TABLE XI (continued)

Medium and Enrichments	Colony Size
Veal Infusion agar horse hemoglobin-serum	Pin-point to 1 mm.
Veal Infusion agar hemoglobin	Pin-point to 1 mm.
Brain Veal agar 2 c.c. Bacto Hemoglobin/plate	Pin-point to 1 mm.
Brain Veal agar 2 c.c. ascites/plate	---------
Heart Infusion agar 2 cc. Bacto Hemoglobin/ plate	---------
Heart Infusion agar 2 c.c. ascitic fluid/plate	---------
North Gelatin agar 2 c.c. Bacto Hemoglobin/plate	0.1-0.5mm.
North Gelatin agar 2 c.c. ascites/plate	---------

(D.S.) indicates doubles trength agar
(S.S.) indicates single strength agar
* Results indicated, but media not used routinely

2 c.c. of enrichment used with 10 c.c. of medium to give a 20% medium

up to 1 mm. in size when ascitic fluid was used in equal proportions with Proteose No. 3 agar (double strength), but they were slightly larger when 2 c.c. of ascitic fluid was used in the single strength agar. With Douglas Chocolate agar, using 2 c.c. of ascitic fluid, no growth was obtained; Bacto Brain Veal agar, Bacto Heart Infusion agar, and Bacto Norths' Gelatine agar with ascitic fluid gave no growth.

Douglas Chocolate agar plates made by adding 10% defibrinated horse blood and heating at 85 C,(other bloods serve as well), gave results comparable to the Proteose No. 3 Bacto Hemoglobin agar (double strength). Bacto Hemoglobin is equally satisfactory in other media where a blood enrichment is called for.

On Peizers' Veal Horse-Hemoglobin-Plasma agar, colonies grew up to 1.5 mm. in diameter, but this medium was not available in quantity. The hemoglobin plasma mixture has no advantage over the other blood enrichments used.

Gonococcus colonies up to 1 mm. in diameter developed on Bacto Brain Veal agar to which 2 c.c. of Bacto Hemoglobin had been added, but failed to grow on this medium when ascitic fluid was substituted for the Bacto Hemoglobin.

Heart Infusion Bacto Hemoglobin agar proved to be unsatisfactory. We have not determined whether the fault lies in the medium or the enrichment substance used.

North Gelatin Bacto Hemoglobin agar gave colonies up to 0.5 mm. in diameter, while ascitic fluid added to this basic

media failed to support growth.

Northe' Gelatin Bacto Hemoglobin agar will support growth, but it does not measure up to the standards set by the Proteose No. 3 Bacto Hemoglobin agar.

Growth-promoting effects of blood charcoal The blood charcoal agar plates were made from Proteose No. 3 agar (double strength), by adding equal quantities of a 1:200 or 1:400 aqueous suspension of charcoal to the medium and letting it harden into plates. When blood was added, the dry powdered charcoal was mixed with it before pouring the plates.

Plates in which the suspension of charcoal was used alone were pure black; but those to which blood was added took on a grayish-black hue.

Most of the plates were not incubated in containers. Instead, wide rubber bands were placed around the outside edge of the petri dish, sealing it off, thus maintaining a constant supply of moisture within the plate.

Due to some factor which we have been unable to determine, the gonococcus grew outside of the incubator at room temperature, the plates containing a mixture of Proteose No. 3 agar (double strength) and charcoal in aqueous solution. The colonies averaged practically the same size as those grown within the incubator at 36.5 C.

Colonies grown on Proteose No. 3 Charcoal Bacto Hemoglobin agar plates, incubated at 36.5 C, varied in size up to 2 mm. in diameter. In some instances the colonies had a grayish tinge

TABLE XII

Study of the Growth Promoting Effects of Blood Charcoal

Medium	Accessory Substances	Carbon Dilution	Activated Charcoal
Proteose No.3 agar (Double Strength)	Bacto Hemoglobin blood charcoal	1:200	Yes
" "	" "	"	"
Proteose No. 3agar (Single Strength)	blood charcoal	"	No
Proteose No. 3agar (Double Strength)	" "	"	"
" "	Bacto Hemoglobin blood charcoal	"	Yes
" "	----------	-----	-----
" " (control)	Bacto Hemoglobin	-----	-----
" "	" "	-----	-----

Temp. of Incubation	Colony Size	Oxidase Test	Appearance		Conditions of Incubation
			colony	media	
36.5 C	0.1-2 mm.	-	ground glass, opaque	gray black	8% CO_2
36.5 C	"	-	" "	"	No CO_2 rubber bands
36.5 C	"	-	gray-white	black	" "
20 C	0.1-0.5 mm.	-	" "	"	" "
36.5 C	0.1 mm	-	" "	"	" "
36.5 C	----	----	-------	clear	" "
36.5 C	0.1-2.5 mm.	-	ground glass, opaque	reddish brown	8% CO_2
36.5 C	0.1-1.0 mm.	-	" "	"	rubber bands

Note: Stock strains were used throughout this experiment.

rather than the typical ground glass appearance. This was due to a variation of strains or some effect created by the charcoal. These strains checked microscopically and reacted typically with the oxidase reagent.

A point not heretofore noted in connection with the use of charcoal, is that even though the media is black, the oxidase reagent can be used to detect the presence of the gonococcus colonies. Before being treated, the colonies are will defined and stand out in relief against the black back-ground, but, close attention must be paid to the color change because colonies can be lost through the blending effect of dye and medium.

The exact nature of the action of carbon in the medium is not known. It may be due to a lowering of the oxygen tension, or to the Fe atom associated with the hemin complex. This complex may enter into an oxidation-reduction reaction in which certain metabolic products of the gonococcus are eliminated, creating more favorable growth conditions.

Some of the carbon was activated at 1000 C for 1 hour in the muffle furnace before it was used. As far as we could determine, the activation had little effect on the colony growth. The organisms grew as will on the inactivated as on the activated. We found that special media to be used with accessory substances were not necessary. In most instances, the addition of the accessory to a simple, well-tested medium, will yield as good results as the special medium.

The fact that the gonococcus will grow on a medium to which blood charcoal has been added seems to indicate that, in blood, the growth factors furnished may not be due so much to the blood itself as to some activating principle in the blood which sets up a series of reactions which are beneficial to the growth of the organism.

In our experiments the gonococcus failed to grow on the Proteose No. 3 agar in the absence of any enrichments. The growth on the controls (Proteose No. 3 Bacto Hemoglobin agar) differed to no noticeable extent from the growth on the charcoal plates.

Colonies grew with equal facility on plates which were incubated in CO_2 and on those incubated without CO_2. The CO_2 in this instance evidently plays a minor role in colony development.

Study of the bacteriostatic effects of certain aniline dyes on the organisms accompanying the gonococcus in the male and female genito-urinary system. Nile Blue A, Crystal Violet, and Basic Fuchsin were added to Proteose No. 3 agar and North Gelatin Bacto Hemoglobin and ascitic fluid were used as enriching substances. The dyes were added so dilutions ranging from 1:25,000 to 1:100,000 were obtained. Material obtained from the clinic was plated out on the media and incubated in a 10% CO_2 tension for 48 hours. The plates were checked for colonies of the gonococcus with the oxidase reagent and smears

were made of all colonies to determine which were Gram positive and negative.

The gonococcus grows in the presence of the dyes used, and in the varying concentrations. Gram positive organisms are inhibited to a considerable degree. None of the dyes used appear to be inhibitory for Streptococcus viridans which is a frequently accompanying organism. In some instances yeast and certain gram positive bacilli were not inhibited. There was no noticeable inhibition of Gram negative organisms.

This particular phase of our work, the study of the action of dyes, was cut short owing to the scarcity of positive material from the clinic.

These experiments were carried out with the available material and the results seem to indicate that there are other dyes in addition to Nile Blue A which can be incorporated in media and used to inhibit Gram positive organisms tending to over grow the gonococcus.

The results with Nile Blue A did not come up to our expectations. Crystal Violet and Basic Fuchsin seem to be as satisfactory if not better than Nile Blue A in their inhibitory action.

The following tables present our findings as thus far completed. The specimens used in these tests were from patients undergoing sulfathiazloe therapy and in many instances no gonococci were present. The accompanying organisms will be designated as Gram negative or Gram positive bacillus,

TABLE XIII

Study of the Inhibitory Action of Nile Blue A, Crystal Violet, and Basic Fuchsin onthe Contaminants Accompanying the Gonococcus

Proteose No. 3 Bacto Hemoglobin agar.

Dilution	g.c. cols.	Col. Size	Inhibition of g.c.	Inhibition for Gm. +	Organisms Prsent
Control	--	--	--	---	Gm +, bac. S. viridans
Crystal Violet					
1:100,000	--	--	--	none for S. viridans	S. viridans
1:50,000	2	2.5 mm.	--	"	"
1:25,000	2	2.5 mm.	--	"	"
Basic Fuchsin					
1:100,000	--	--	--	none for Gm. pos. bac. spreader	Gm. - bac.
1:50,000	3	2.5 mm.	--	none for S. viridans	SViridans
1:25,000	2	2mm.	--	"	"

Dilution	g.c. cols.	Col. Size	Inhibition of g.c.	Inhibition for Gm +	Organsims Present
Nile Blue A					
1:16666	--	--	--	none for Gm + bac. spreader	Gm.- bac. Gm. + bac.
1:8333	1	1mm.	--	none for S. viridans	S. viridans
1:4166	1	1mm.	--	none for S.Viridans Staph	S. aureusi St. viridans

Proteuse No. 3 agar with ascitic fluid was totally unsatisfactory in the above experiment. The only organsim that gave satisfactory growth on it were Staph. aureus, a gram positive bacillus, and a gram positive cocco-bacillus. Viridans did not grow on the ascitic-proteose No. 3 combination.

TABLE XIII(cont.)

The Relative Numbers of Gm. + and Gm.- Colonies on Plates in Which Dyes Were Used

Medium	Control		Basic Fuchsin 1:25,000		1:50,000		1:100,000		Crystal Violet 1:25,000		1:50,000		1:100,000		Nile Blue A 1:4166		1:8333		1:16666	
Gram Stain	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+
Prot. No. 3 Bacto-Hemo.	50		7	5	3	1	6	12	4	1		1	3	2	20		20		30	
" "	20	1	8		16		15		40		40		21		42		60		32	1
North Gelatin Bacto Hemo.	41	3	40		30		30		50		20		30		25		30		20	

staphylococcus, etc. The degree of colony inhibition will be noted on the charts.

Observations on the oxidase reaction Both the dimethyl and tetramethyl compounds were used. Owing to the toxicity of the dimethyl compound its use was discontinued in favor of the tetramethyl which was less toxic. One percent aqueous solutions were used. The tetramethyl para-phenylene diamine hydrochloride tended to be absorbed by most mediums. The stained area was often so dark, that identification of colonies from the purplish-black background was difficult.

Superiority of culture over smear methods of diagnosis Though no data were routinely kept on the superiority of the culture over smear methods of diagnosis, it has been our general experience that the advantages of the culture method far outweighs any other method of diagnosis thus far presented.

SUMMARY

Many types of media have been proposed for the cultivation of the gonococcus, but the greater number of these media require special ingredients and are difficult to prepare in the small laboratory.

The question has arisen as to whether or not the gonococcus is constant in its staining reactions and morphology. Some investigators are inclined to believe that Gram positive variants occur. We are unable to either deny or verify this point, but we do believe that many of the variants reported are contaminants or the results of incorrect reporting on improperly stained specimens.

Temperature has been shown to play an important role in the incubation of the gonococcus, but from our observations we are inclined to differ with Neumann(1937) and Winkler(1937) who claim that the temperature of both medium and inoculum are factors in the isolation and cultivation of this organism.

We agree with the majority of investigators that the gonococcus is carbondioxidophillic, at least during primary isolation. The optimum temperature for its cultivation is 36.5 C, though an occasional strain is encountered which requires a lower temperature of incubation.

A solid medium with a moist surface is more satisfactory

than a semi-solid medium. A moist surface is not absolutely necessary, since it has been shown that the gonococcus will grow on culture plates which have been exposed to the drying action of room temperatures for periods as long as 43 days without noticeable effect on the growth of the organism.

Ascitic fluid has been recommended as a satisfactory enriching substance for culture plates. It has been our experience that it is generally inferior to blood enrichments. In our work the dessicated Bacto Hemoglobin yielded the best results, although most whole bloods were satisfactory. Bacto Hemoglobin is more convenient to keep on hand and prepare in the small laboratory.

Previous investigations have shown that blood is an excellent accessory substance. The mechanism involved in the production of the growth stimulating factors is not thoroughly understood. The work of Glass and Kennet (1939) on the action of blood charcoal added to culture media in place of whole blood seems to indicate that the mechanism is tied up in the Fe present in the hemin nucleus. Our experiments with blood charcoal seem to indicate that when mixed with Proteose No. 3 agar the growth of the gonococcus is comparable to growth on regular Proteose No. 3 Bacto Hemoglobin agar. The catalytic agen present is of suffieient strength to give satisfactory growth on plates incubated at room temperature.

The aniline dyes, nile blue A, basic fuchsin, and crystal violet were added to Proteose No, 3 agar and North Gelatin agar in varying dilutions. The gonococcus will grow in the presence of crystal violet and basic fuchsin in dilutions varying from 1:25,000 to 1:200,000, and in the presence of nile blue A in diltions from 1:4200 to 1:16666. The Gram positive organisms were largely inhibited. This phase of our work was prematurely cut short due to a shortage of suitable culture material from the clinic.

The only other study of culture media, to determine the most reliable for routine laboratory use, was made by Mak (1936). We are inclined to discount portions of his work since several of the media he used, including Vedder's starch agar, have since proven unreliable for routine culture proceedures.

The results of a study of six different standard culture media recommended for isolation and cultivation of the gonococcus indicate that Proteose No. 3 Bacto Hemoglobin agar and Douglas Chocolate agar are the most reliable media available for routine work. Proteose No. 3 agar is the easiest to prepare and keep in the laboratory for long periods of time.

The ideal medium is one which is clear, simple in composition, and will yield consistent results. This, we believe, is an impossible combination in the existing culture media for the gonococcus. The main difficulties lie in the lack of an accessory substance, which when added, will give a clear, satisfactory medium, and in the staining action of the oxidase reagent which often makes it impossible to distinguish between co

colony and medium.

Of the oxidase reagents used, only one, the tetra methyl p-phenylene diamine hydrochloride proved satisfactory. The mono- and di-methyl derivatives were too toxic for the gonococcus. The stained organisms failed to survive when transplanted.

Though no definite data were kept on the superiority of culture over smear methods of diagnosis, it has been our general experience that the culture method is far superior to any other method thus far presented.

Under the section on experimental work, various laboratory methods were presented in illustrated form. This was done because it was felt that too little emphasis has been placed on descriptive methods in the literature.

CONCLUSIONS

1. The gonococcus is definitely carbondioxidophillic during primary isolation. An optimum temperature of 36.5 C is necessary for most strains, though an occasional strain is encountered which requires a lower temperature.

2. The temperature of media and specimen is not a factor in the isolation of the gonococcus. Inoculations made at room, incubator, or refrigerator temperatures are equally satisfactory.

3. The gonococcus will grow on a dry, moisture-free surface provided the plates are incubated in a CO_2 tension in closed containers. There were no noticeable variations between those colonies grown on moisture free(kept at room temperature for 43 days) and on moist plates.

4. Blood charcoal supplies some accessory suitable for the growth of the gonococcus. From our observations on the growth and size of the colonies, it appears that the action is due to some catalytic agent contained in the charcoal which comes from the blood. This may be due to the Fe in the Fe-hemin complex which remains in the charcoal after the blood has been reduced to the carbon form.

5. A simplified method of introducing carbon dioxide into culture jars was presented. This method and apparatus is simpler and easier to operate than any we have worked with.

6. We have presented methods and proceedures for the inoculation and cultivation of the gonococcus which we feel are simple, accurate and uniform.

7. Of all the media and accessory substances subjected to tests, we found that Douglas Chocolate agar (using whole bloods) and Proteose No. 3 Bacto Hemoglobin agar gave by far the most consistent results. Proteose No. 3 agar proved best for all purposes, including,general cultivation and isolation of the gonococcus, as an agar base for dyes, and with blood charcoal. The dessicated Proteose No. 3 agar is simple to prepare and keep in the laboratory for long periods of time. Douglas agar is difficult to prepare and keep on hand. Colonies on Proteose No. 3 Bacto Hemoglobin agar developed larger and there was less growth of contaminants than on any other medium tested. We feel that it is the best medium available today.

8. Three aniline dyes were tested on Proteose No. 3 agar and on North Gelatin agar. Nile blue A, basic fuchsin, and crystal violet are bacteriostatic to many Gram positive organisms associated with the gonococcus in the male and female urogenital systems. In concentrations of 1:200,000to 1:25,000 with crystal violet and basic fuchsin the gonococcus was not inhibited . Nile blue A in concentrations of 1:4166 to 1:16666 was not inhibitory to Gram negative organisms. Positive specimens were difficult to obtain, with the result that this phase of our investigation could not be completed at this time.

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