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TITLE Optimal Carbon Dioxide Tensions For Primary Isolation Of The Gonococcus; Response Of The Organism To Other Gaseous Environments

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A Thesis

Submitted to the Faculty of Michigan State College of Agriculture and Applied Science in partial fulfillment of the requirements for the degree of Doctor of Philosophy

by

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OPTIMAL CARBON DIOXIDE TENSIONS FOR PRIMARY ISOLATION OF THE GONOCOCCUS; RESPONSE OF THE ORGANISM TO OTHER GASEOUS ENVIRONMENTS

Since the successful cultivation of the gonococcus by E. Bumm in 1885 (1) a vast amount of literature has appeared on the growth requirements and cultivation of this organism. Of these observations a comparatively small part has dealt with the atmospheric requirements of gonococci. Until recent years reports on optimal gaseous environment have been contradictory and based, in some instances, on a study of a comparatively few strains.

The growing mass of evidence indicates that gonococci are benefited during primary isolation by an atmosphere enriched with carbon dioxide. There has grown up among bacteriologists a feeling, based on inconclusive evidence, that to achieve optimal results one must supply a carbon dioxide tension of approximately ten per cent. The methods in current use for supplying carbon dioxide enrichment undoubtedly give varying amounts of the gas, and a study (2) of growth under carbon dioxide-tensions supplied by various means indicates that comparable results may be achieved.

During the course of experiments on the viability of gonococci under conditions of transportation from patient to laboratory (3), the writer used various means of supplying carbon dioxide for culture. The results were practically the same by two methods which were judged to provide different amounts of gas. Analyses confirmed our opinion that the gas tensions supplied were considerably different.

With this information as an impetus, the present study was undertaken to define more exactly the carbon dioxide requirements of gonococci during primary isolation and, also, to examine the effect of gaseous environments other than carbon dioxide.

LITERATURE

Wherry and Oliver (4) in 1916 called attention to the fact that the gonococcus is a partial oxygen-tension organism. In producing a partial oxygen tension they made use of a method employed by an earlier worker in cultivating Brucella abortus. By means of rubber tubing they fastened together an agar slant tube on which gonorrheal material was inoculated and another slant tube containing a transfer of B. subtilis. The gas exchange between the tubes took place through the cotton plugs. Wherry and Oliver felt the successful growth of the gonococcus was due to the lowering of the oxygen tension by Bacillus subtilis. Similar inoculum under atmospheric conditions failed to yield growth of gonococci.

During an investigation of the cultural requirements of the gonococcus, Chapin (5) tested the influence of carbon dioxide. He felt that the carbon dioxide tension present in living tissues, which he gave as five to seven cm. of mercury, was the logical tension to employ in growing gonococci. In isolating the cocci from urethral exudate, Chapin found that a carbon dioxide tension of approximately ten per cent caused a more vigorous growth of the organisms than normal air. The gas was supplied by treating sodium bicarbonate with sulfuric acid. Chapin stressed the importance of a moist atmosphere. He mentioned, in his brief article, that a candle had been found to supply sufficient carbon dioxide for good growth.

Ruediger (6), reporting on the successful recovery of gonococci from three cases of gonorrheal infection, announced that a vigorous growth was obtained by excluding the air from culture tubes by means of rubber stoppers.

Schwartz (7) found, similarly, that excluding air from culture tubes

gave better results than incubating the gonococci under atmospheric conditions. By heating the tops of his tubes in the flame before stoppering, he secured a reduction in atmospheric pressure of about ten per cent. He concluded, also, that moisture is essential for growth.

Rockwell and McKhann (8) have been quoted in the literature as asserting that growth of gonococci is facilitated by the presence of hydrogen gas. This statement is likely to be misleading unless it is understood that the primary isolations of these workers were obtained in atmospheres altered by growth of Bacillus subtilis. In studying the adaptation of gonococci to various gaseous environment they found that cultures isolated under partial oxygen tension grew in pure hydrogen but not in carbon dioxide and oxygen. After growth for some time under atmospheres of hydrogen the organisms seemed to become more sensitive to the inhibiting influence of carbon dioxide and oxygen, but growth was still excellent under partial tension. They found that when strains isolated at partial tension would not grow under pure carbon dioxide or pure oxygen, they could be adapted to do so.

In 1922 Erickson and Albert (9) reported their experiences in cultivating gonococci. Like the earlier workers they stressed the importance of moist media and moist atmosphere. They tested the methods described by Wherry and Cliver and others for producing a reduced oxygen tension and found no advantage over the oxygen tension of normal air. They concluded that the satisfactory results of investigators using reduced oxygen tension was due to an increased amount of moisture brought about by the technique employed to exclude air.

In the same year Torrey and Buckell (10) published an account of

extensive work on the primary isolation of gonococci. Like Erickson and Albert they found a reduced oxygen tension to prove in no way superior to moist air of normal pressure. Enrichment of the atmosphere with carbon dioxide according to Chapin's method likewise yielded no better results than normal air.

The introduction of the direct oxidase reaction as an aid in identifying the gonococcus in mixed culture, by McLeod, Coates et al. (11) in 1934, focused the attention of workers on the general cultural methods of the English investigators. McLeod and his colleagues endorsed the use of an increased carbon dioxide tension of eight per cent, incubating their cultures under the enriched carbon dioxide atmosphere for 24 hours, followed by incubation in normal air for 24 hours. Their choice of a carbon dioxide tension of eight per cent resulted from incubating gonorrheal material under tensions of four, eight and sixteen per cent atmospheres of carbon dioxide. In a short series of specimens, the results appeared to be best in the middle amount, to quote the authors. As this approximated the ten per cent recommended by Chapin it was adopted for routine work. According to their observations old stock strains of gonococci were not aided in their growth by carbon dioxide. The effect of an atmosphere of hydrogen was also tried on primary culture, in a few instances, but no worthwhile advantages were observed.

The observations of McLeod and his associates in regard to optimal atmospheric conditions are of interest.

"The general impression left by these observations (those summarized above) is that there are some strains of gonococcus which are

favored in their growth at the outset by an atmosphere of raised carbon dioxide content. This may be due to need for some delicate adjustment of reaction which is most easily developed in a carbon dioxide atmosphere or to the fact that the gonococcus is a micro-organism more highly adapted than most to its gaseous environment and one which, therefore, grows best at first in a concentration of carbon dioxide approximating that which exists in the tissues."

Leahy and Carpenter (12) introduced the methods of McLeod and his co-workers to this country with some modifications. They used an ingenious manometric method of measuring the carbon dioxide supplied by tank to sealed culture jars. Of 61 strains of gonococci isolated under a ten per cent carbon dioxide tension, only 52 grew in a jar in which the gaseous environment had not been altered. In this series the relative humidity in the two culture jars was supposedly the same. Whether or not the authors tested other concentrations of carbon dioxide before adopting an enrichment of ten per cent is not indicated.

Spink and Keefer (13), like Chapin, found that a candle burned in the culture jar facilitated primary isolation of gonococci.

Christensen and Schoenlein (2) cultured 122 clinic specimens from gonorrheal patients in multiple platings, subjecting each set of plates to the following conditions:

(1) Carbon dioxide supplied by candle

(2) Carbon dioxide (approx. 10 per cent) supplied by placing moistened oats in the closed container.

(3) Carbon dioxide supplied by action of sulfuric acid on sodium bicarbonate

(4) 8-10 per cent carbon dioxide supplied by tank

(5) Moist air

The number of strains isolated by the respective methods were:

(1) 71 (2) 69 (3) 70 (4) 70 (5) 64

They noted also that pure cultures of the gonococcus previously isolated do not require carbon dioxide for growth.

During the years 1935 and 1936 some interesting work was published in Germany on the atmospheric requirements of the gonococcus. Neumann (14, 15) investigated the ability of primary strains of gonococci to grow under aerobic and anaerobic conditions, reduced oxygen t ension and increased carbon dioxide tension. To obtain anaerobic conditions he used the method of Fortner (16) with slight modifications. Bacterium prodigiosus was the organism used to consume oxygen. The anaerobic method was abandoned after the observation that gonococcus colonies in the Fortner plate attained their maximum size in 24 hours, whereas a strict anaerobe, inoculated along with the gonorrheal material, first became visible at about this time. Neumann reasoned that the gonococci developed during a period of reduced oxygen tension and ceased growth when anaerobic conditions were attained. He termed the anaerobic plate the "B" plate method.

To obtain reduced oxygen tension, or partial vacuum, Neumann inverted the medium-containing half of a Petri dish on a glass plate of 13 x 13 cm. dimensions which had been heated in a flame. The Petri dish was sealed in place by plasticine. This method he called the "C" plate method. He determined, by means of a sensitive water manometer, that a pressure decrease continued in uninoculated "C" plates during incubation. This decrease he attributed to the reducing action of the agar. Reduced oxygen tension in the "C" plate, therefore, was attained by heating the glass plates and by action of the medium.

For the carbon dioxide enriched, or "D" plate method, Neumann placed an inoculated half of a Petri dish on an unheated glass plate, but pushed a piece of dry ice under the dish before molding the plasticine in place. According to the author, "The carbon dioxide volume necessary for gonoccus growth may be about ten per cent. Such a large volume is not necessary, however. Through numerous comparative experiments I have found empirically, without measurement, that a piece of dry ice the size of a pea supplies all requirements. ...with a piece of dry ice the size of a bean there may develop enough carbon dioxide to inhibit the growth of gonococci."

Using the aerobic, or "A" plate, the "C" and "D" plate, Neumann obtained the following results in a total of 161 strains of gonococci isolated: 113 grew aerobically; 30 grew under reduced oxygen tension and carbon dioxide; 18 grew under carbon dioxide only. However, all strains grew under carbon dioxide.

Winkler (17) used the technique of Neumann and concluded that there are aerobic gonococci which grow only on the "A" plates; that there are gonococci which grow only on the "C" or "D" plates; there are also strains which can grow on all plates without regard to oxygen content. The ratio in which these three groups of strains exist to one another is about 1:1:1. The best methods of cultivating the gonococcus are normal and carbon dioxide enriched atmospheres.

Since the work of McLeod, Coates et al (11), there is agreement among investigators that carbon dioxide enriched atmospheres facilitate growth of most strains of gonococci. There is agreement, also, that a percentage of approximately ten produces the best results. It is interesting to note why the different workers arrived at that percentage.

In the case of Chapin (5) who preceded McLeod, the tension of the carbon dioxide in living tissues influenced his choice. According to him the tension varies from five to seven cm. of mercury. His figures are at variance with those of Campbell (18) who determined the carbon dioxide tension in subcutaneous tissue and in the pleural and peritoneal cavities of man as equivalent to four to five cm. of mercury. In normal urine of man Campbell found the tensions to be the same. Transposed into percentages, the tensions determined by Campbell would be from 5.3 to 6.6; the tension as stated by Chapin would be from 6.6 to 9.2 per cent.

McLeod and his colleagues cite the work of Chapin and the successful cultivation of Brucella abortus under ten per cent carbon dioxide as reasons for investigating the effect of this gas. It has been stated before that these workers fixed on eight per cent as optimum, after a short series of comparisons. Leahy and Carpenter (12) investigated the work of McLeod and adopted ten per cent as the percentage of choice, possibly from unpublished data. Neumann (14, 15) apparently made no measurement of the carbon dioxide furnished in his "D" plates but judged it to be about ten per cent. It is probable that the work of the foregoing investigators influenced Neumann in naming a ten per cent carbon dioxide tension as the desirable concentration.

In this country the methods of culture advocated by Carpenter are widely used. In his section on "The Gonococcus", "Diagnostic Procedures and Reagents," 1941 (19), Carpenter advises the use of a carbon dioxide enriched atmosphere which may be obtained in three ways:

(1) 10 per cent by tank with approximate determination of gas tension by manometer

(2) By burning a candle in the culture jar before placing on the lid

(3) By use of $NaHCO_3$ and H_2SO_4

One infers that equally good results may be obtained by all of these methods.

Since, according to Christensen and Schoenlein (2), the candle method provides adequate growth stimulation of gonococci as well as eight to ten per cent carbon dioxide supplied by tank, the logical first step in this investigation was the determination of the carbon dioxide tension supplied by candle.

GAS ANALYSES

The culture jars used were heavy brass cylinders fitted with inlet and outlet nipples in the lids. The lids were made air-tight by means of soft rubber gaskets and wing nuts. The cylinders were originally designed for modified Fildes-MacIntosh anaerobic jars, as described by Cummings (20), but served excellently for gonococcus culture. By test they were found capable of holding a partial vacuum of 40 cm. of mercury for 48 hours, as measured on a U-type open manometer. The total capacity was approximately six liters.

In preparing the jars for test, a "smokeless" candle was placed in the bottom of the container and lighted. The candle was allowed to burn five minutes before the jar lid was fitted in place. Burning the candle for varying lengths of time before putting on the lid made no marked difference in the carbon dioxide content furnished. The jars were allowed to stand overnight or some hours before testing in order for the gases to come to room temperature. At 20 to 24 degrees centigrade it was noted that a partial vacuum existed in the candle jars.

This partial vacuum when measured on the open manometer was found to vary between four to seven cm. of mercury.

A modified Henderson-Haldane apparatus was used in preliminary carbon dioxide and oxygen determinations, but constant results were not obtained with this device. The possible sources of error appeared to be due in part to the apparatus and in part to faulty manipulation. The sample of ten cc. of gas which the Henderson apparatus tests, and the slow absorption of both carbon dioxide and oxygen by potassium hydroxide and alkaline pyrogallol, respectively, are factors which tend to cause error. After repeated tests with different jars in which the volumes varied slightly, the average percentages of the gases were determined as:

Carbon dioxide	1.75 to 1.85
Oxygen	17.5 to 17.23

It was brought to our attention shortly after these preliminary tests that Nye and Lamb (21) had made determinations of the carbon dioxide and oxygen content of sealed museum jars in which a candle had been burned. These determinations were made in the course of study on the effect of carbon dioxide tension in the primary isolation of streptococci, meningococci and gonococci from pus and body fluids. No mention was made by the authors of the apparatus used in obtaining the following percentages of gases:

	Before Incubation	After 48 hours
Small candle jar (12 x 20 cm.)		
per cent carbon dioxide	1.74	1.85
per cent oxygen	17.87	18.00
Large candle jar (15 x 30 cm.)		
per cent carbon dioxide	2.91	3.08
per cent oxygen	16.76	16.56

It will be noted that the carbon dioxide tension determined by Nye and Lamb was higher than that obtained by our measurement. The large candle jar in the above table had a capacity of about 5300 cc., while the jars used in this study varied from 5900 cc. to 6000 cc.

Because of this discrepancy in determinations it was decided to substitute an apparatus using an ascarite train for the KOH absorption bottle of the Henderson-Haldane analyser.

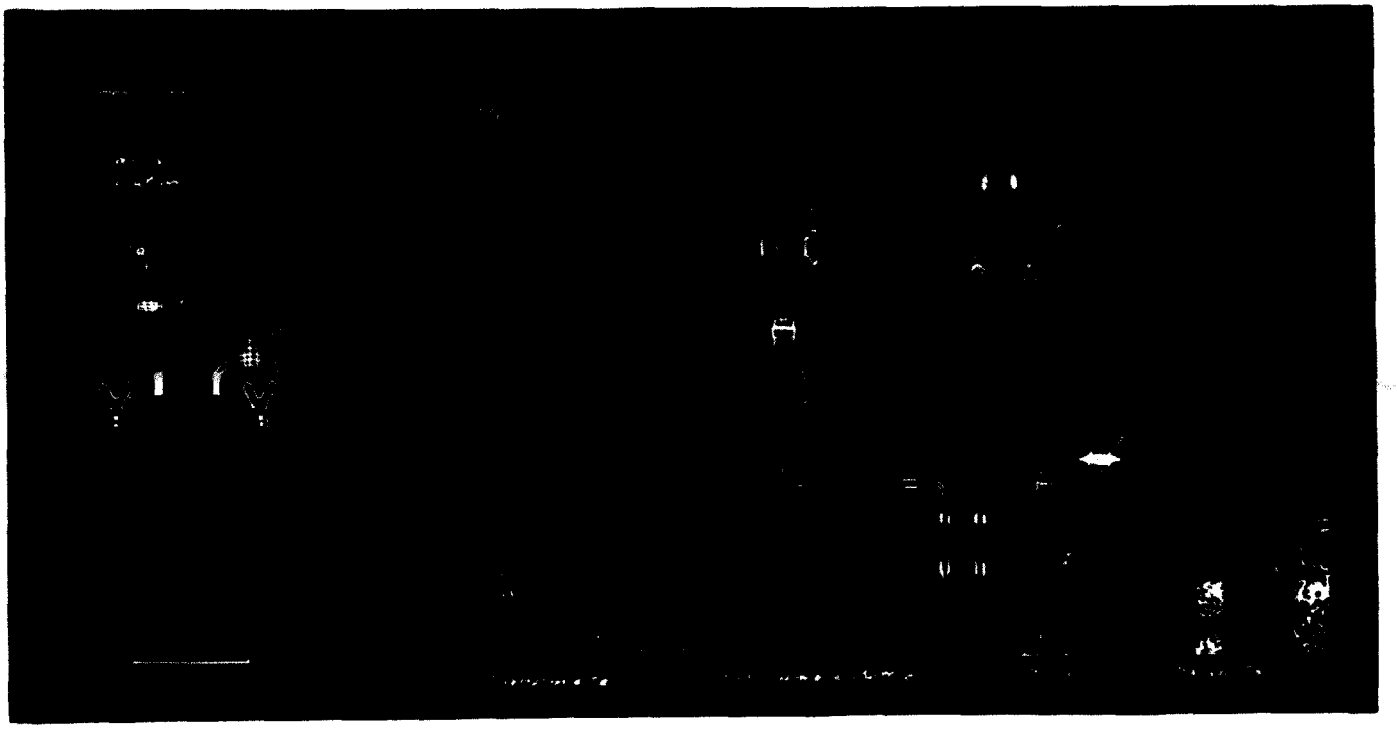


Figure 1

The diagram given above shows the ascarite apparatus with its various units set up for carbon dioxide determination.

In operation, valve B was opened and the two-way stopcock"C" turned to connect the 150 cc. sample bottle with the candle jar. The mercury reservoir was lowered until the mercury in the sample bottle

reached the 150 c.c. mark on the glass shank sealed to the bottom of the sample bottle. A slight vacuum was then indicated on the open manometer. Calcium chloride solution* was allowed to flow into the candle jar until the mercury columns of the manometer were level. Stopcock C was then turned to connect the sample bottle with the tube leading to D, a bottle of concentrated sulfuric acid.** A two-way stopcock E was opened to the air. The mercury reservoir was raised, forcing the gas in the sample bottle to bubble through the acid in D. Two 150 cc. portions of gas were forced through D before a sample was sent through the ascarite train. This was done to clean the acid bottle and the tubing of previous gas samples. In sending a gas sample through the train the operations just described were repeated, except that stopcock E was turned to connect D with the ascarite bottles F and G. The glass valves in F and G were opened and the gas then forced through the ascarite. Bottle "F" was weighed on an analytical balance and the increase in weight due to absorption of carbon dioxide was determined by subtracting final weight from initial weight. Bottle "G" was used to eliminate absorption of carbon dioxide from air in the test train. This precaution was felt to be necessary since a partial vacuum developed in the ascarite bottles.

Calculation of the per cent of carbon dioxide in a sample was made by multiplying the weight of carbon dioxide in grams by the conversion factor 509. This value in cubic centimeters was divided by 150, the volume of the sample. Correction for barometric pressure and temperature

* 40% calcium chloride solution. Carbon dioxide is approximately 1/16 as soluble in this solution as in water.

** The sulfuric acid was used to remove moisture from the gas.

was made and this value multiplied by 100.

The percentages of carbon dioxide determined by this method were found to vary slightly, but very close agreement was obtained in samples from the same jar. The range was from 2.25 per cent to 2.35, with the average 2.29 per cent.

PLAN OF THE STUDY

The concern of workers interested in demonstrating the effect of gaseous environment has been, chiefly, with the problem of how many strains of gonococci in a series of specimens could be isolated under carbon dioxide, atmospheric air, or other conditions. From the literature it appears that a wide difference exists between the growth-stimulating properties of carbon dioxide and other gaseous conditions. Therefore, no close estimation of the populations maintained by the different environments has been necessary.

Our purpose, as stated at the beginning of the paper, has been to define the optimal limits of carbon dioxide stimulation as well as to investigate the effect of gases other than carbon dioxide. A plate count as a means of arriving at the relative population sustained by different percentages of carbon dioxide seemed a necessity. It was felt that this same method of estimation could be extended to the growth of gonococci under reduced oxygen tension and other conditions.

The difficulty of counting an organism which cannot be treated by the pour-plate technique was realized, as well as the possible errors of the dilution method. However, our experience in the culture of the gonococcus had taught us that a count was possible on surface platings, and it seemed reasonable to suppose that any considerable

difference in population could be detected.

It was realized that to arrive at an estimation of the limits of optimal carbon dioxide stimulation a fairly large population of gonococci must be present in each specimen. For that reason culture of acute cases only was planned and carried out.

The study resolved itself into these parts:

Part I	Comparison of the effect of atmospheric air, reduced oxygen tension and carbon dioxide.
Part Ia	Study of the effect of moisture on "aerobic" strains of gonococci.
Part II	Determination of the optimal range of carbon dioxide stimulation.
Part IIa	Effect of carbon dioxide beyond the optimal range.
Part III	Effect of hydrogen and carbon monoxide compared with atmospheric air, reduced oxygen tension and carbon dioxide.
Part IIIa	Effect of nitrogen compared with atmospheric air, reduced oxygen tension and carbon dioxide.

PART I. COMPARISON OF THE EFFECT OF ATMOSPHERIC AIR, REDUCED OXYGEN TENSION AND CARBON DIOXIDE

The work of Neumann (14, 15) and Winkler (17) demonstrated that reduced oxygen tension obtained by partial vacuum contributes to the growth of some strains of gonococci. The high moisture content of the small "C" plates used by these workers may account, in part, for the success of this method over the aerobic condition. (It was noted by Neuman that in the open incubator gonococci grew better in stormy weather than in clear.) It is probable that a small amount of carbon dioxide produced by attendant organisms also contributed to growth in the "C" plates.

Before attempting to define the limits of optimal carbon-dioxide stimulation in this study it was felt necessary to observe the effect of reduced oxygen tension, since this condition necessarily accompanies enrichment with carbon dioxide; also it was desireable to learn the effect of moisture, since a higher moisture content is present in the closed containers than in the open incubator.

It was judged that incubation under the following conditions would permit a comparison of the effect of reduced oxygen tension and carbon dioxide tension:

Carbon dioxide, ten per cent, supplied by tank.

Partial vacuum equal to nine cm. of mercury on an open manometer. (This reduced oxygen tension is equivalent to the lowered tension created by introducing ten per cent carbon dioxide into a culture jar by the manometeric method of measurement.)

Carbon dioxide, 2.29± per cent, furnished by burning a candle in an air-tight container.

Partial vacuum equal to seven cm. of mercury on an open manometer. (It was observed that a partial vacuum varying from four to seven cm. of mercury was created by the burning of a candle in a closed jar. The mean of these figures plus the 1.5 cm. (approx.) of mercury, representing 2 per cent carbon dioxide, equals seven cm.)

Carbon dioxide furnished by burning a candle in a container whose pressure came to equilibrium with atmospheric air.

Atmospheric air.

METHODS

The description of materials, and technique which follows applies,

for the most part, to the entire study. Where a method applies only to Part I, mention has been made of the fact.

MEDIA

The chocolate agar of Difco was used as the plate medium. The ingredients for the agar base were:

Proteose Peptone #3.....2.0 per cent

Bacto-dextrose.....0.05 per cent

Sodium chloride.....0.5 per cent

Disodium phosphate.....0.5 per cent

Two grams of Bacto-hemoglobin, a dehydrated product from washed beef blood corpuscles, were dissolved in 100 cc. of distilled water at 50° C. The partial solution was filtered through coarse moistened cheesecloth to remove undissolved particles and then autoclaved for 20 minutes at 121° C.

The agar base and sterile solution of hemoglobin were cooled to 50-55° C. and mixed. Plates were poured with this mixture, approximately 20 cc. to the plate.

The chocolate agar plates were always prepared on the day they were to be inoculated. It was felt that the moist plates more than compensated for occasional contaminants introduced during preparation.

Dilution medium: A sterile two per cent solution of proteose-peptone #3 and 0.5 per cent sodium chloride were used in all dilutions of the original inoculum suspension. The pH of this solution was 7.3.

Collection medium: Sterile ascitic fluid, undiluted, was adjusted to a pH of approximately 6.2 by bubbling Carbon dioxide gas through it. The fluid was distributed in 2 cc. amounts in sterile 3 x 3/8" tubes and the tubes were rubber-stoppered.

Collection Swabs: These were the usual absorbent cotton swabs rolled tightly on wooden applicator sticks. The amount of cotton was about one-fourth that used in the ordinary throat swab. It was possible with a small swab to enter the male anterior urethra without brushing the surface of the glans penis.

Collection of specimens:

With the exception of one specimen the material was obtained from male patients at the Venereal Disease Clinic maintained by the Detroit Department of Health, Detroit, Michigan. In order to obtain a moderate to large population of gonococci in each specimen, exudate was taken from acute cases only. Male cases of gonorrhea were selected since it is possible to isolate nearly pure cultures of gonococci from the exudate; in the material from a female the vaginal flora frequently overgrows the gonococci and makes an enumeration of the population impossible.

Collection of specimens was made by the author in the male diagnostic clinic. After a diagnosis of "acute gonorrhea" was pronounced by the examining physician, exudate was obtained from the patient by cotton swab and the swab washed off in the collection medium of ascitic fluid. The swab was carefully pressed to the sides of the tube, then discarded. The tube was labelled with the patient's clinic number and stoppered. From the Clinic Laboratory results of slide examination on exudate from each patient were obtained. Only specimens from acute cases in which a laboratory report of "Gram negative extra- or intra-cellular diplococci found" were retained for culture. The specimen tubes were packed for transportation in a large thermos bottle containing cracked ice.

Since transportation from the Venereal Disease Clinic in Detroit to the laboratory in Lansing involved a drive of two and a half hours,

the iced thermos bottle was found to be a necessity in preventing overgrowth of secondary organisms in the collection fluid. This was particularly true in summer weather. Often a time interval of six hours elapsed from the taking of specimens to inoculation of plates. However, the number of secondary organisms in a specimen was usually small and the bacteria were kept in a nearly static condition.

No effort was made to check on the loss in gonococcus population in specimens transported under these conditions. A loss occurred, undoubtedly, but the original number of organisms in practically all specimens was sufficiently great to insure a count after culture.

Dilution and Inoculation of Specimens:

Some indication of the dilutions to be made on a particular specimen were obtained from observation of the number of organisms present in smears examined at the clinic. A specimen whose smear was heavy with gonococci was diluted more than one which showed a few organisms per field. Specimens heavy with gram-positive cocci or bacilli were seldom cultured. Occasionally observation of smears was not possible and only the results of microscopic examination could be obtained.

It was found by experiment that the following range of dilutions would make possible a count on practically all specimens:

Direct inoculation from collection fluid.....0.1 cc. inoculum

1-10 dilution.....0.1 cc. inoculum

1-20 dilution.....0.1 cc. inoculum

1-100 dilution.....0.1 cc. inoculum

1-200 dilution.....0.1 cc. inoculum

In most cases 0.1 cc. inoculum direct and from a 1-100 dilution were sufficient to obtain a sparse population for counts. The aim not

always achieved, was to secure plates in some dilution with a count less than 150.

Because of mucous threads and pus cells present in the exudate, the collection fluid had to be shaken vigorously to insure an even distribution of organisms and extraneous matter. Dilutions were made as follows, since the collection fluid was 2 cc. or less:

0.5 cc. (c.f.) + 4.5 cc. of diluent = 1-10

0.2 cc. (c.f.) + 3.8 cc. of diluent = 1-20

0.1 cc. (c.f.) + 9.9 cc. of diluent = 1-100

4 cc. (1-100 dilution) + 4 cc. of diluent = 1-200

Only one set of dilutions was made per specimen. Therefore, plates of a 1-100 dilution, for example, to be incubated under various atmospheric conditions were inoculated from the same dilution tube. Inoculation of a series of plates was made with a 1 cc. pipette graduated in tenths.

It was found that sterile bent glass rods gave a satisfactory spread of material. No difficulty was experienced in covering the entire surface of the slightly moist plates with 0.1 cc. inoculum.

PROVISION FOR DIFFERENT ATMOSPHERIC CONDITIONS, Part I

Plates to be incubated aerobically were placed in a large glass desiccator whose top was pushed slightly to one side to allow exchange of air. Five hundred cc. of water were placed in the bottom compartment ordinarily occupied by the desiccant. A Taylor "Humidiguide" which had been calibrated against wet and dry bulb thermometers was used to test the atmosphere in this container. On repeated tests the relative humidity was indicated as 60 to 70 per cent.

Plates to be incubated under partial vacuum or carbon dioxide were

placed in the brass jars described under "gas analysis." One hundred fifty cc to two hundred cc. of water was placed in wide-mouthed bottles in each of the containers. No measurement was made of the relative humidity in the closed jars; however, from the large amount of condensation which was visible on the inner sides and tops of the jars following incubation, it is probable that the moisture concentration was close to saturation.

One jar was enriched with ten per cent carbon dioxide by the method described by Leahy and Carpenter (12). By means of rubber tubing a tank of medical carbon dioxide, a mercury open U-tube type of manometer, the culture jar and the vacuum line were connected to each other in the order named. Air was evacuated from the jar until the pressure was reduced by nine cm. of mercury (removal of approximately 12 per cent of air). Carbon dioxide was allowed to flow into the jar until the manometer reading was 1.5 cm. below atmospheric pressure. The partial vacuum of 1.5 cm. allowed for gas expansion at incubator temperature.

Two jars were partially evacuated until the pressures on the U-tube manometer were, respectively, nine and seven cm. of mercury below that of atmospheric air.

Candles were placed in two jars, the candles lighted and allowed to burn five minutes before the jar lids were clamped in place. One jar was air-tight and the other had a slow leak around the rubber gasket which permitted a gradual adjustment of pressure equilibrium with the outside air. Both jars were tested on the manometer after incubation. A partial vacuum of four to seven cm. of mercury could always be demonstrated on the air-tight container.

Incubation temperature. Throughout the study all cultures were incubated for approximately 48 hours at a temperature varying from 35 to 36° C., according to the temperature limits advised by Carpenter (19). Fluctuations in temperature were marked by a recording thermometer, and a check was made at intervals with a mercury thermometer.

EXAMINATION OF THE PLATES FOLLOWING INCUBATION

Approximately 48 hours after inoculation of the plates, the jars were opened and the plates examined for gonococcus colonies. Growth usually consisted nearly exclusively of convex, transparent colonies with undulated margins, typical of the gonococcus. Colonies of secondary organisms were relatively few and these were usually staphylococci, green-producing streptococci or coliform bacteria. Proteus was encountered on a few plates.

All plates were sprayed by a DeVilbiss "atomizer" with a 1 per cent solution of para-amino-dimethyl-aniline monohydrochloride (Eastman). Excess dye solution was drained from the plates.

This dye component in the presence of the oxidase of the gonococcus produces a pink colony. On further oxidation the color becomes maroon and finally black. The speed of the color change is not constant with different strains of gonococci but the shift from pink to maroon to black usually takes place within 12 to 15 minutes with all strains. Organisms in a colony are viable at the pink stage but non-viable when oxidation of the dye is complete. The dye does not interfere with subsequent Gram stains. The direct oxidase reaction with para-amino-dimethyl-aniline monohydrochloride is not peculiar to the gonococcus but is given by the whole Neisseria genus, as well as Hemophilus

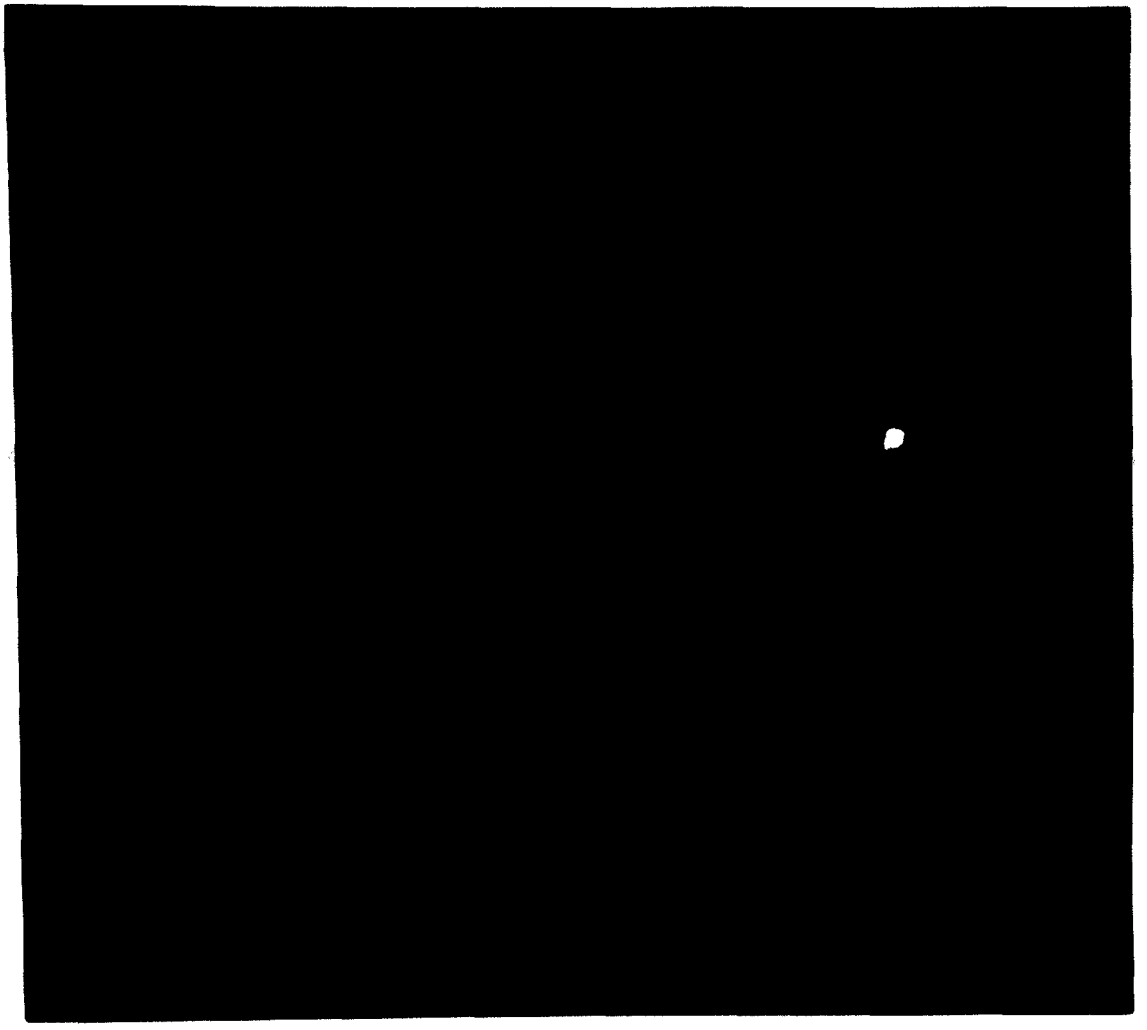
influenzae, certain gram-positive diplobacilli, the cholera vibrio and members of the alkaligenes group (11, 12, 19). Gronau (22) mentions colonies of certain diphtheroid-like organisms which give an oxidase-positive reaction. The color of the colony after oxidation is a chalky, grayish black. Organisms similar to these were found in this study but were easily differentiated from gonococcus growth.

A gram stain was made of two oxidase-positive colonies on each plate selected for counting. The preparations were examined under the microscope for gram-negative, coffee-bean-shaped diplococci.

A count was made of typical oxidase-positive colonies on plates which had been determined by macroscopic examination of the colonies and gram stain to contain gonococci. Wherever possible a count was made of every plate in a dilution series, provided the growth was not crowded. Crowded plates with a count above 200 were considered "innumerable" if higher dilutions in the same series were countable. It is possible that occasional colonies of bacteria other than gonococci were counted by this all-inclusive method. However, from the very few stained slide preparations which contained other organisms than gram-negative diplococci, it is probable that errors of this kind were few. Carpenter's experience would lead us to believe that the occurrence of any other Neisseria than the gonococcus in cultures from the male urethra is rare. According to him (19), "It is significant that during the nine-year period the author has made cultures of gonococcus, only three cultures of Neisseria other than gonococcus have been recovered from the lower birth canal and from the genito-urinary tract of men." Recently Carpenter has had to amend that statement somewhat. Carpenter

and Charles (23) have reported isolation of meningococci from the genito-urinary tract of seven patients with clinical symptoms of gonorrhea. These strains are the first to be reported in the literature from such a source, and it is felt by the authors that their occurrence is rare.

Comparison of Counts (Methods)



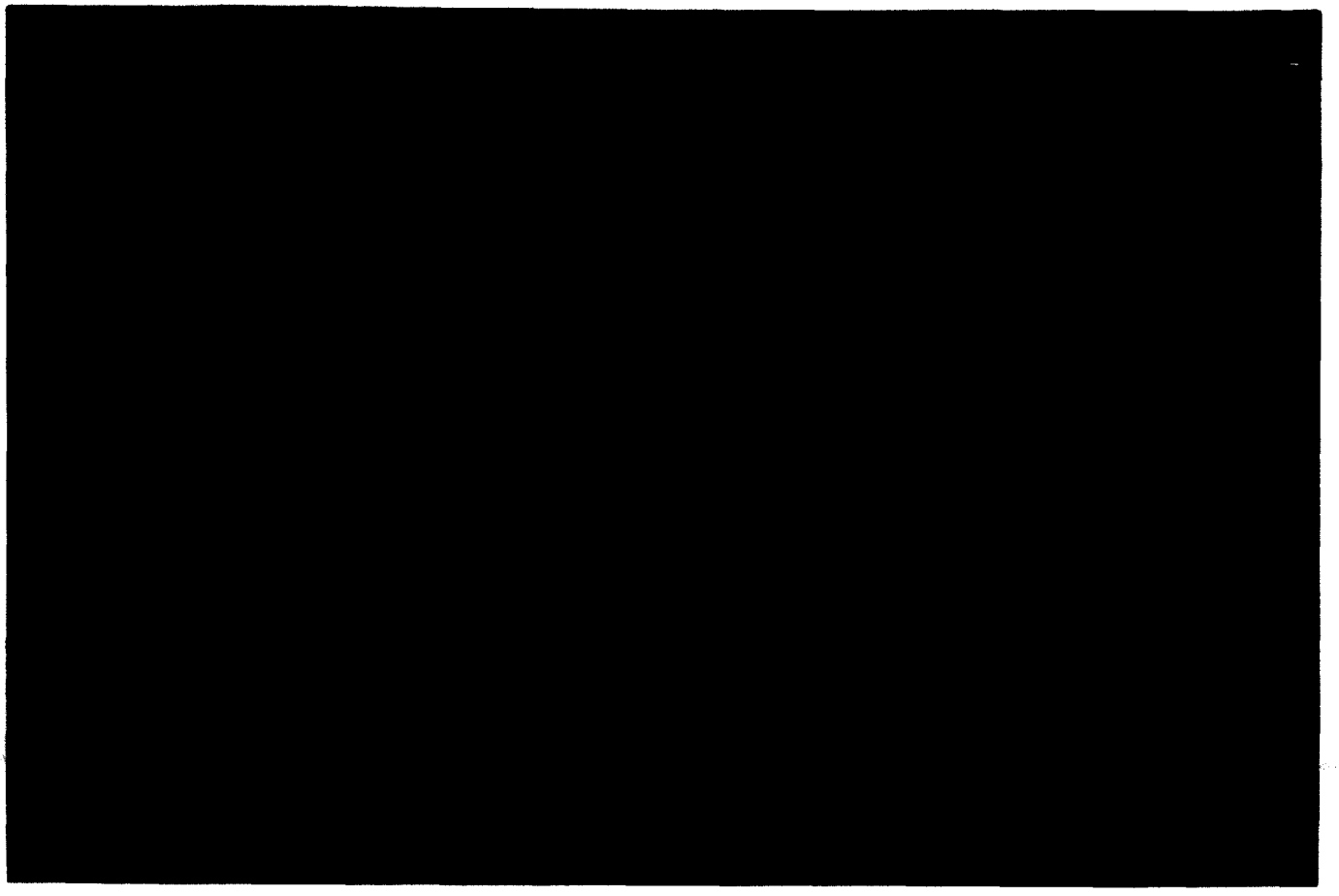
The figures in Table 1 are taken from counts made on cultures one, two, and four (7-31-41) and are representative of the results in Part I.

No effort was made in totalling the counts to arrive at the number of organisms grown from 1.0 cc. of inoculum, as the conventional count is made from poured plates. A comparison of numbers only was sought in whatever dilution permitted a comparison. For example, the comparative count was easily obtained from Culture #1. The number of gonococci observed on the various "direct" plates was taken as representative of the reaction of Culture #1 to different atmospheric conditions. The numbers 4, 98, 15, 90, 18, and 21 were assigned to the respective conditions.

A comparative count for Culture #2 was more difficult. The aerobic growth was zero; growth on the three carbon dioxide conditions could be compared readily; a comparison of the counts on the partial vacuum plates was easily made. However, a comparison of "partial vacuum" counts and those on the carbon dioxide plates could be made only by approximating the "innumerable" carbon dioxide plate from its count on a higher dilution.

A comparative count for culture four was easily made since the organisms grew under carbon dioxide only. Comparison could be obtained on the 1-10 dilution plates.

Results of Part I



Out of some eighty cultures which grew under one or more of the conditions recorded in Table two, 68 were suitable for counting. Of the 68 strains considered, all grew under ten per cent carbon dioxide and 67 grew under carbon dioxide supplied by candle in an air-tight container. The one strain which grew under ten per cent carbon dioxide and not under "candle" was from a clinical case of gonorrhea. Intracellular gram-negative diplococci were present on a slide taken at the time exudate was obtained from the patient for culture. Two oxidase-positive colonies only were present on the culture plates.

Only 58 strains developed in the "open" container in which a candle had been burned. It was hoped that this condition in which carbon dioxide was present, but no partial vacuum, would indicate the part played by the four to seven cm. partial vacuum present in an air-tight candle jar. The results were inconclusive, since it is probable that loss of carbon dioxide through gas exchange was responsible for the comparatively poor results of the "open" container, rather than heightened oxygen tension.

Better results were obtained under partial vacuum than by aerobic incubation. The number of strains isolated under the former condition was greater by only four than the number grown aerobically, but the difference in population was considerable.

On the second line of the table are recorded the comparative populations of 38 strains which grew aerobically and under all of the other conditions. The comparison has been made in percentages to facilitate its reading. The total population obtained under ten per cent carbon dioxide has been given the arbitrary value of 100, since growth was best under this condition. The percentage values of the other counts were obtained, of course, by dividing the total counts of 38 strains grown aerobically, etc., by the total count obtained under ten per cent carbon dioxide and multiplying by 100. It will be noted that growth stimulation by the various conditions was in the following descending order: ten per cent carbon dioxide, candle in air-tight container, candle in "open" container, partial vacuum 7 cm., partial vacuum 9 cm., aerobic condition. The difference between the growth supported by the first two conditions and the last four was very great.

On line three a comparison is made of 42 strains which grew under reduced oxygen tension, ten per cent carbon dioxide, and carbon dioxide furnished by candle in the air-tight container. Again the population under ten per cent carbon dioxide was greatest, with the candle supplying nearly the same growth stimulation. Growth under reduced oxygen tension was less by nearly 70 per cent than that produced under carbon dioxide.

Finally a comparison is made of the growth obtained under ten per cent carbon dioxide and the candle in "closed" container. A reversal of the previous order will be noted. Probably due to the chance of random sampling, growth was greater for 67 strains under candle conditions than for 68 strains under ten per cent carbon dioxide.

PART IA

The poor growth of gonococci under the aerobic condition in the preceding experiment may be attributed in part to atmosphere and in part to the moisture concentration of that atmosphere. In an effort to determine the effect of moisture, a series of specimens was cultured in duplicate under the following conditions:

(1) The aerobic condition of Part I, relative humidity 60-70 per cent

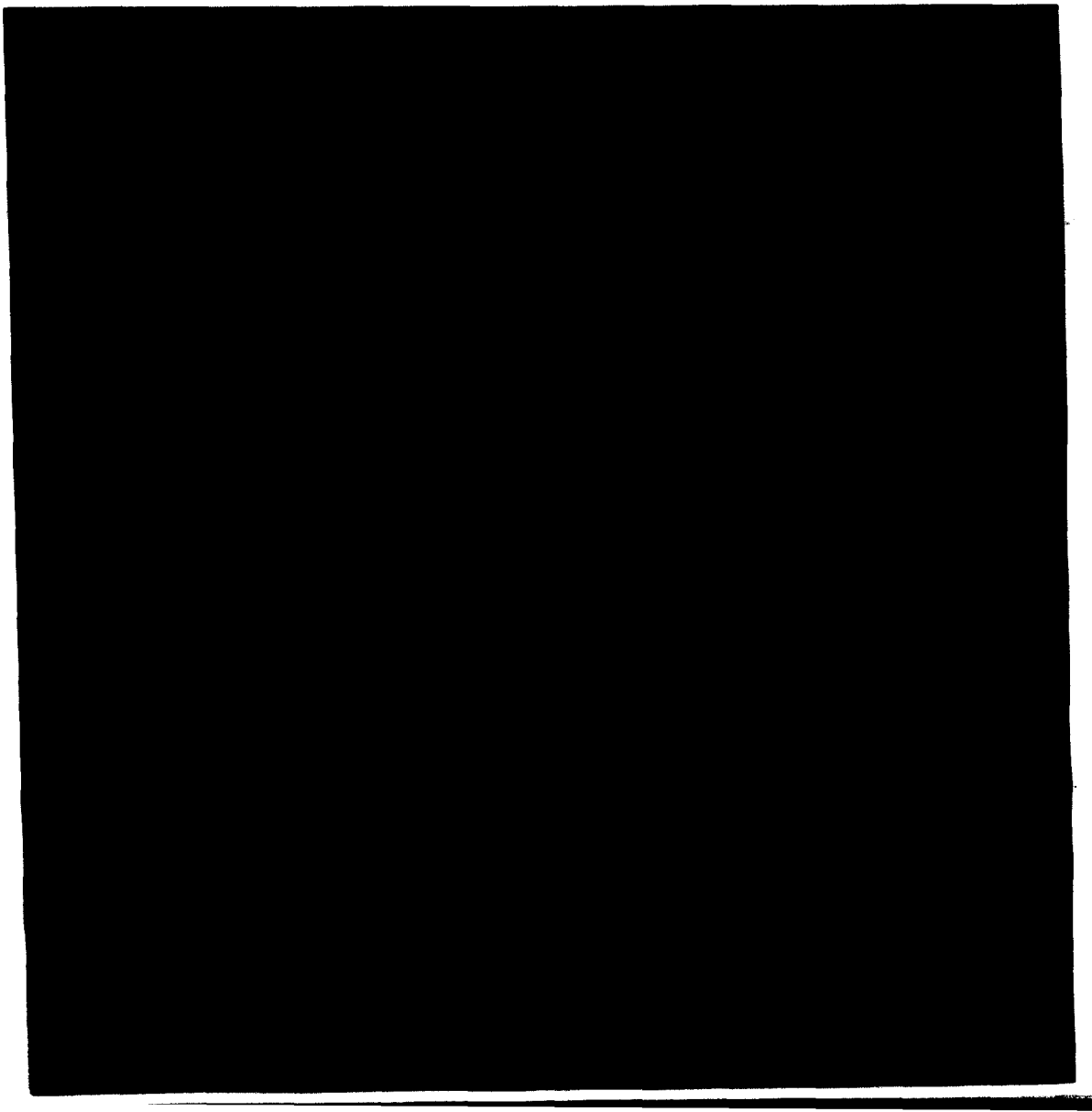
(2) Atmospheric air in a closed container, relative humidity approximately 100 per cent

(3) Ten per cent carbon dioxide, supplied by tank, relative humidity approximately 100 per cent

The carbon dioxide atmosphere was included only for purposes of comparison. It was recognized that the conditions of (2) could not be

called true aerobiosis, since growth of gonococci and any secondary organisms in the specimens would alter the carbon dioxide and oxygen tensions. However, in a large container this effect is probably small and obtains in any of the carbon dioxide enriched or reduced oxygen tension conditions.

A total of 24 specimens were cultured, 13 of which were aerobic. The 13 strains are considered in Table 3. Duplicate platings were made of undiluted and diluted collection fluid under each condition. The inoculum consisted of 0.1 cc. of undiluted collection fluid and 0.1 cc. of a 1-100 dilution. Counts on the duplicate plates were made whenever growth was sufficiently sparse for a count.



With all 13 strains of gonococci a greater population was obtained in the saturated atmosphere than in the 60-70 per cent relative humidity. The effect of moisture apparently varied with the strain. For example, with culture #1 the growth was many times greater under a saturated atmosphere than under 60-70 per cent relative humidity. With culture #8 the growth was not markedly different under the two conditions.

Even though the 13 strains were aerobic, the growth was in every instance greater under carbon dioxide than on the aerobic plates. If further confirmation of the beneficial effect of carbon dioxide were needed, this protocol would appear to supply it.

PART II. OPTIMAL LIMITS OF CARBON DIOXIDE STIMULATION

The results in Part I correspond to those of Christensen and Schoenlein (2) in that the number of strains of gonococci isolated under carbon dioxide supplied by candle is about the same as the number isolated under ten per cent carbon dioxide. In our experiment the amount of growth obtained by both methods was practically the same. It would appear, therefore, that one may supply carbon dioxide in percentages varying from about two to ten per cent and expect growth to be the same under all tensions.

In an experiment to define the optimal limits of carbon dioxide stimulation, two per cent carbon dioxide supplied by tank was chosen as the smallest amount of gas to be tested and 22 per cent was selected as the maximum. Enrichment by carbon dioxide of the same or slightly less tension than that supplied by candle was felt desirable, since other gases than carbon dioxide are produced by burning of a candle, particularly carbon monoxide. Twenty-two per cent carbon dioxide was chosen

as the maximum tension simply as a convenient limit for preliminary tests.

The following atmospheric conditions were provided:

(1) Atmospheric air in open container similar to that used for aerobic growth in Part I. Relative humidity, 60-70 per cent.

(2) Carbon dioxide supplied by candle in an air-tight container. Relative humidity approximately 100 per cent.

(3) Carbon dioxide, two per cent supplied by tank. Relative humidity approximately 100 per cent.

(4) Carbon dioxide, six per cent supplied by tank. Relative humidity approximately 100 per cent.

(5) Carbon dioxide, 10 per cent supplied by tank. Relative humidity approximately 100 per cent.

(6) Carbon dioxide 14 per cent supplied by tank. Relative humidity approximately 100 per cent.

(7) Carbon dioxide, 18 per cent supplied by tank. Relative humidity approximately 100 per cent.

(8) Carbon dioxide, 22 per cent supplied by tank. Relative humidity approximately 100 per cent.

Measurement of the carbon dioxide supplied by tank was by the manometric method described under "Methods." Calculation of the amount of air to evacuate from the jars was made as follows:

For a final 14 per cent enrichment of carbon dioxide, for example:
760 mm. x 0.14 = 106 mm. or 10.6 cm.
10.6 cm. + 1.5 cm. (partial vacuum to provide for expansion) = 12.1 cm.

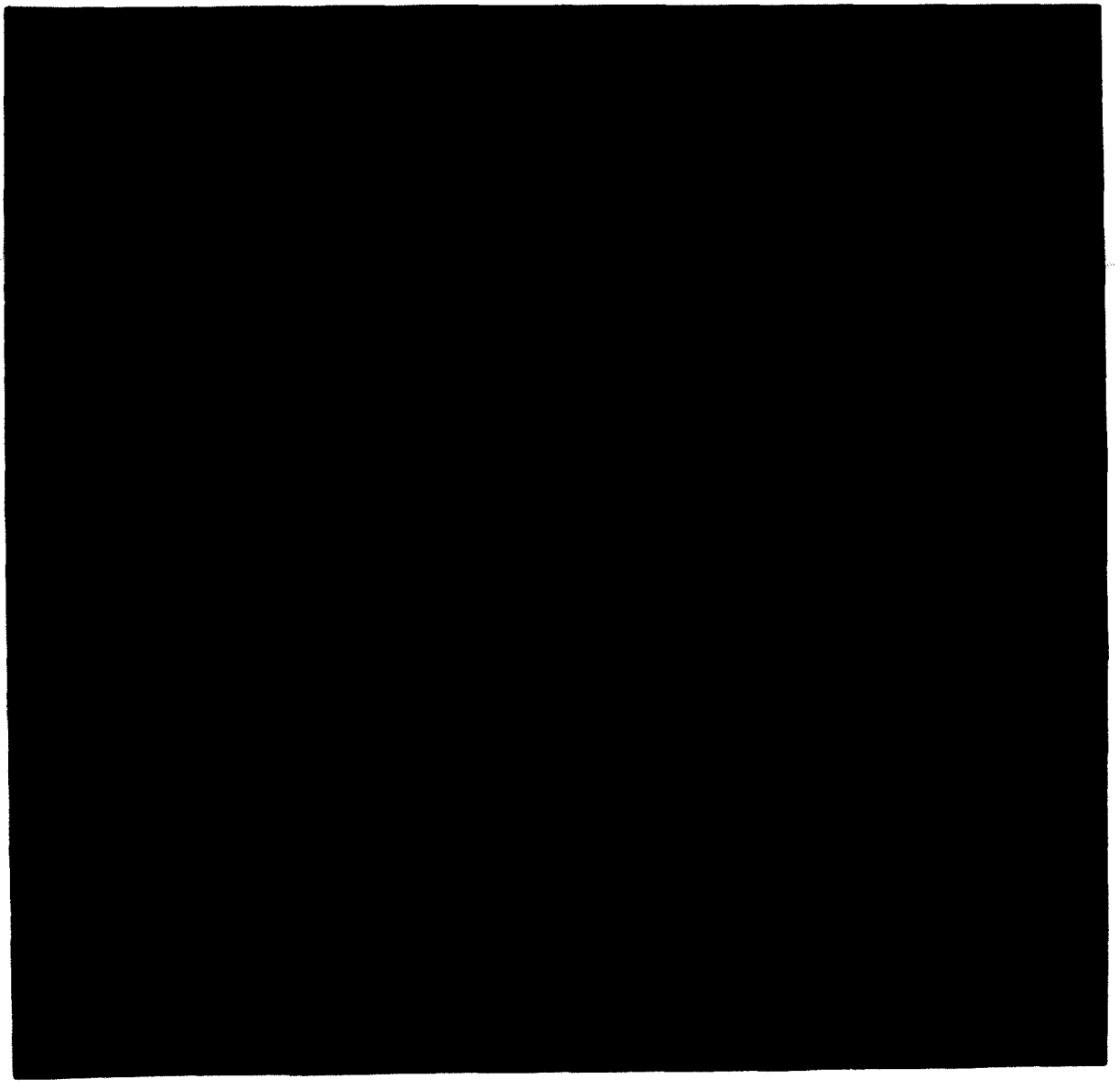
Air was evacuated until the pressure was reduced by 12 cm. of mercury; then gas was allowed to flow in until the manometer reading was 1.5 cm. below atmospheric pressure.

Gas analyses were made on jars filled by this method with amounts of carbon dioxide varying from two to 22 per cent. No corrections were made for normal temperature and pressure at the time of filling. The analyses demonstrated that the actual amount of gas present varied as much as 0.5 to 1.0 per cent from the calculated amount. This was not felt to be a

serious error, since a wide range of carbon dioxide tension was under test; furthermore, a difference of four per cent existed between each condition.

The methods and technique of Part I were employed. A single set of dilutions was made from each specimen. Eight plates were inoculated directly from the collection fluid with 0.1 cc. inoculum, and eight plates were inoculated from each of the dilutions. The duplicate plates were marked with specimen number, dilution and atmospheric condition and placed in their respective jars.

The plates after examination for gonococci were counted as in Part I. A comparative count only was sought. The results are tabulated in Table 4.



A surprising uniformity will be noted in the total counts and the number of strains grown under the following conditions: "Candle", six per cent carbon dioxide, ten per cent carbon dioxide, 14 per cent carbon dioxide, and 18 per cent carbon dioxide. At the extremes, under atmospheres of two per cent and 22 per cent carbon dioxide, the count was less than under the other carbon dioxide tensions. Growth under the aerobic condition was so much less than on duplicate plates grown under carbon dioxide that no attempt was made to arrive at a comparison in population. The heaviest growth was obtained on plates incubated in "Candle," and ten per cent carbon dioxide atmospheres, probably due to chance in sampling. Only thirty-one strains were isolated under ten per cent carbon dioxide, yet the total growth was greater than in plates incubated under any other carbon dioxide tensions but that provided by candle.

Whether or not any significance might be attached to the lower counts obtained under two per cent carbon dioxide and 22 per cent carbon dioxide could be determined only by further culture work. It was decided that a wider range of carbon dioxide tensions might aid in indicating at what tension inhibition occurs and stimulation ceases.

For the second series of Part II, the following conditions were provided:

(1) Atmospheric air in an open container similar to that used for aerobic growth in Part I. Relative humidity, 60 to 70 per cent,

(2) Carbon dioxide supplied by candle in an air-tight container. Relative humidity approximately 100 per cent.

(3) Carbon dioxide, two per cent supplied by tank. Relative humidity approximately 100 per cent.

(4) Carbon dioxide, six per cent supplied by tank. Relative humidity approximately 100 per cent.

(5) Carbon dioxide, 10 per cent supplied by tank. Relative humidity approximately 100 per cent.

(6) Carbon dioxide, 14 per cent supplied by tank. Relative humidity approximately 100 per cent

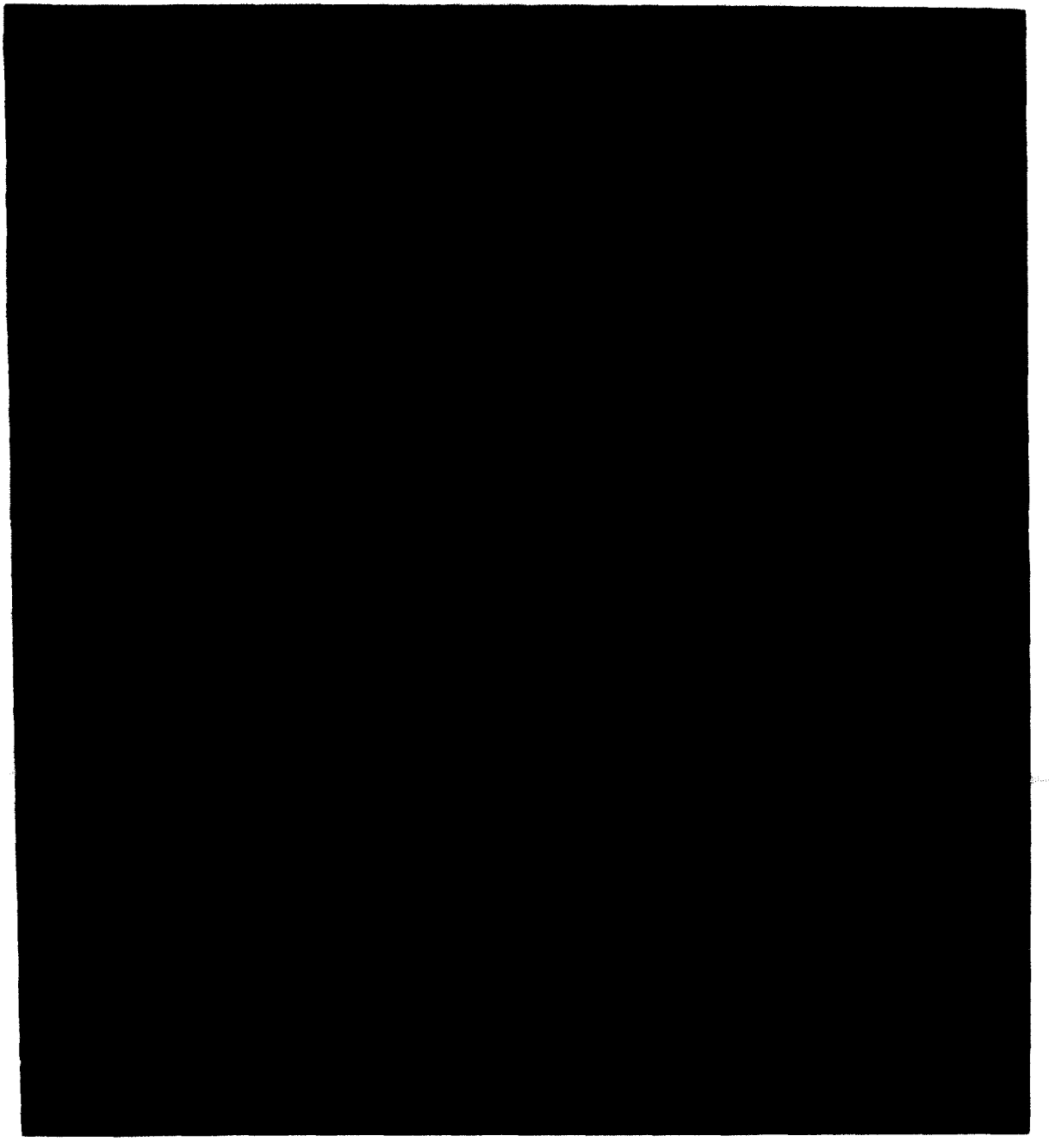
(7) Carbon dioxide, 18 per cent supplied by tank. Relative humidity approximately 100 per cent.

(8) Carbon dioxide, 22 per cent supplied by tank. Relative humidity approximately 100 per cent.

(9) Carbon dioxide, 26 per cent supplied by tank. Relative humidity approximately 100 per cent.

(10) Carbon dioxide, 30 per cent supplied by tank. Relative humidity approximately 100 per cent.

In the second series, 27 strains of gonococci were countable on some or all of the plates. The results are given in Table 5.



Aerobic growth, as usual, was considerably less than growth under carbon dioxide. Only 14 of the 27 strains under consideration grew aerobically.

As in the preceding series, fairly uniform results were obtained under "candle," 6 per cent carbon dioxide, 10 per cent carbon dioxide,

14 per cent carbon dioxide, and 18 per cent carbon dioxide. The relationship of the various counts expressed in percentages is as follows:

Carbon dioxide, 10 per cent.....100 per cent

"Candle"..... 96

Carbon dioxide, 6 per cent..... 94

Carbon dioxide, 18 per cent..... 93

Carbon dioxide, 14 per cent..... 89

Carbon dioxide, 2 per cent..... 85

Carbon dioxide, 22 per cent..... 79

Carbon dioxide, 26 per cent..... 65

Carbon dioxide, 30 per cent..... 62

Maximum growth stimulation was obtained with carbon dioxide tensions ranging from 2.3 to 18 per cent (with the inclusion of the "candle" condition). While 27 strains grew under 22 per cent carbon dioxide it will be noted that the population obtained under this condition was considerably less than that of the condition below it in the series, 18 per cent carbon dioxide. It would appear that in this series, as in series 1, stimulation of gonococci decreased somewhat above the tension of 18 per cent carbon dioxide and the decline was sufficiently marked to be determined by plate count at a tension of 22 per cent carbon dioxide. The decline in growth stimulation, or increase in inhibition, was even more marked under 26 and 30 per cent carbon dioxide.

Wilson (24) has pointed out that to obtain a small distribution error in the plate count method, a dilution giving the proper number of

colonies per plate (100-400) must be available in order to minimize crowding of colonies and error in sampling.

Wilson's observation, while applicable to poured plates in which the major portion of the growth is subsurface, is difficult to apply to the surface-plating method used in this study. The size of the individual gonococcus colony ranges from 0.5 mm. to 2.0 mm. and some crowding occurs with a count above 150. When the count is above 200, considerable crowding takes place and an approximation only can be made.

In the comparison experiments of Part II, and throughout this study, dilutions have been made with the intention of providing plates with counts of 150 or less: first to permit easy counting, secondly to permit recognition of typical colonies. In about 70 per cent of the counts the number of colonies per plate has been 150 or less; frequently the number has been less than 100.

Wilson and Kullman (25) determined that further reduction of distribution error may be made by providing a sufficient number of duplicate plates, three to five.

It was recognized by us that the single plate count is subject to errors, yet sufficiently accurate to detect fairly wide differences in population. In order to test the validity of the indications obtained by the single plate count as to optimal range of carbon dioxide stimulation, an experiment was made in which the significant atmospheric conditions of Part II were repeated and five duplicate plates were inoculated for each condition.

The exudates from six cases of acute gonorrhea were cultured under the following conditions:

Carbon dioxide, 2 per cent, supplied by tank

Carbon dioxide, 10 per cent, supplied by tank

Carbon dioxide, 18 per cent, supplied by tank

Carbon dioxide, 22 per cent, supplied by tank

Exudates from six other cases of acute gonorrhea were cultured under:

Carbon dioxide, 2 per cent, supplied by tank

Carbon dioxide, 10 per cent, supplied by tank

Carbon dioxide, 18 per cent, supplied by tank

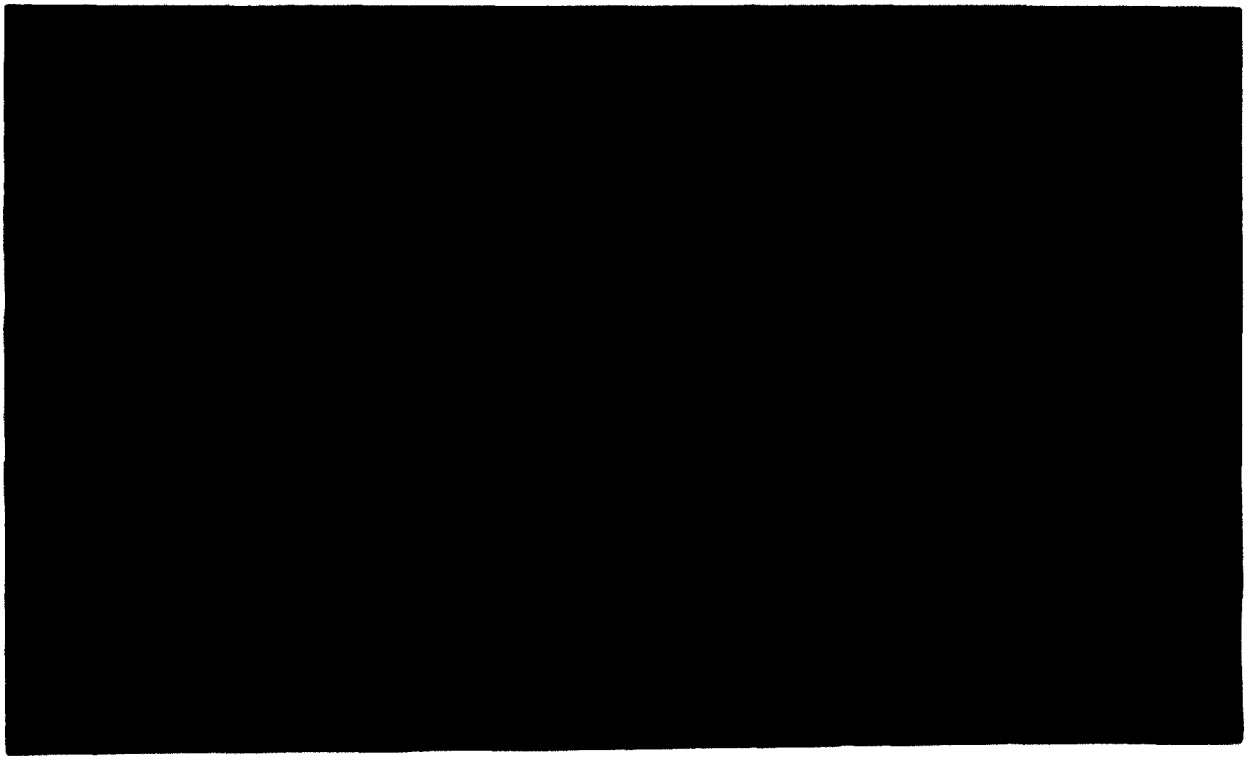
Carbon dioxide, 22 per cent, supplied by tank

Carbon dioxide, 30 per cent, supplied by tank

The relative humidity in each culture jar was close to saturation. As in previous experiments one set of dilutions was made from each specimen to eliminate errors which might arise from using a series of similar dilutions. With 11 specimens a 1:20 and 1:100 dilution were made; with one specimen direct inoculum from the collection fluid and 1:20 dilution were utilized. A total of 20 plates was inoculated from each dilution tube, or the collection fluid, and the inoculum spread by glass rod.

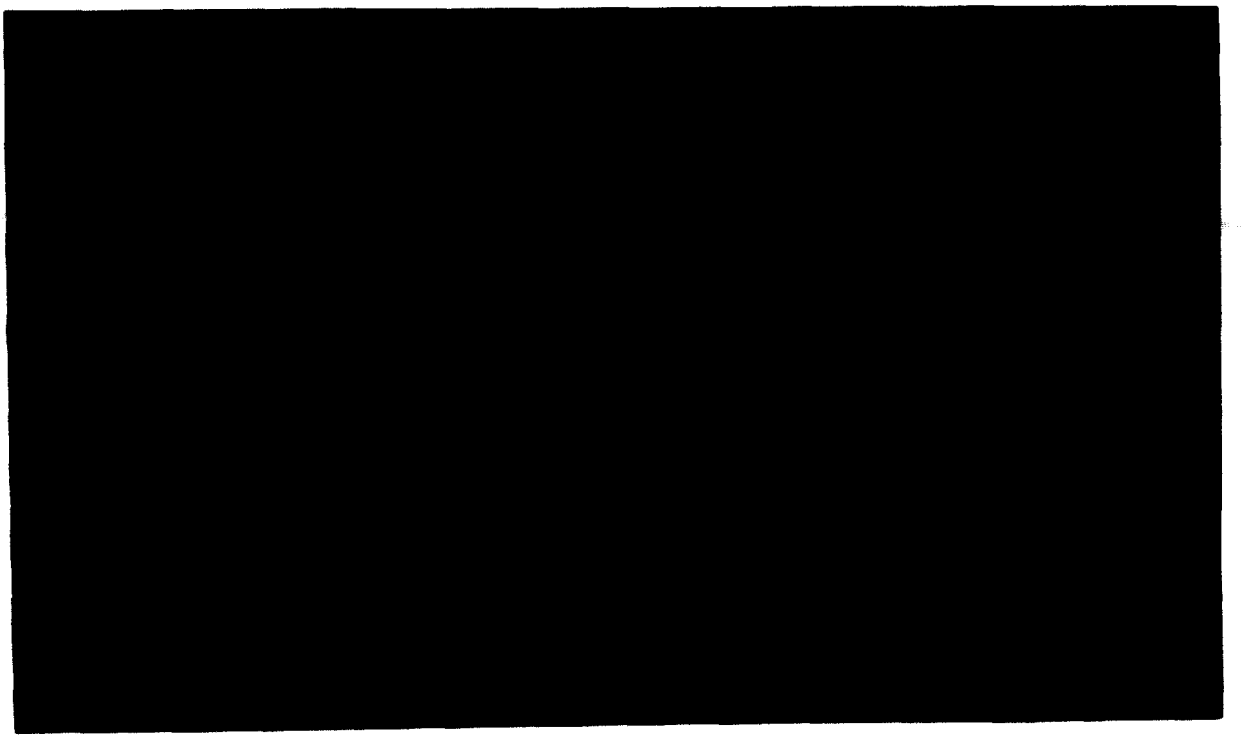
In Table 6 the average of the counts from five plates is recorded under each condition. The figures given are the average of the count in one dilution only, in seven instances the 1:100 dilution, in five instances the 1:20 dilution.

In the above table, series 3, the total counts and percentage comparisons indicate that six cultures of gonococci grew uniformly under two per cent, ten per cent, and 18 per cent carbon dioxide. Growth was considerably less under 22 per cent carbon dioxide than under the other tensions.



The six cultures considered in Table 6, series 4, grew best under 18 per cent carbon dioxide and 10 per cent carbon dioxide. A decided drop in population occurred under two per cent and 22 per cent carbon dioxide. The drop in population was even more pronounced under 30 per cent carbon dioxide. These results follow closely those obtained with the single-plate counts.

A comparison of the percentage relationships between the total counts obtained by the single-plate and five-plate count methods brings out clearly agreement and discrepancy. The comparison is given in Table 7.



An examination of Table 7 will reveal that, with one exception, the counts obtained by single-plate technique were confirmed by the five-plate method. The exception will be found in Table 6, series 3, under

two per cent carbon dioxide. Growth under two per cent carbon dioxide slightly exceeded in this one series growth under ten per cent and 18 per cent carbon dioxide. There are several possible explanations for this exception.

1. For the six cultures in this series, two per cent carbon dioxide represented the optimum atmospheric condition.
2. Errors in sampling raised the count.
3. The concentration of carbon dioxide gas in the culture jars was greater than two per cent.

Of the three possibilities we are inclined to favor the last. Gas analyses established that carbon dioxide concentrations varied as much as 0.5 to 1.0 per cent when the culture jars were filled by tank. While this difference would not be serious in the higher ranges of six and 18 per cent carbon dioxide, an error of 0.5 to 1.0 per cent might affect considerably the growth-promoting qualities of a concentration approaching the lower limit of the optimal zone.

The evidence in series 1, 2 and 4, Part II indicates that two per cent carbon dioxide enrichment is below the optimum, while the data of Parts I and II indicate that the concentration of carbon dioxide furnished by candle is within the zone of optimal growth stimulation. If we accept, tentatively, the idea that growth stimulation by a candle burned in an air-tight container is due to carbon dioxide gas alone (excepting the factor of moisture), it is evident that the lower limit of the optimal zone of carbon dioxide stimulation for gonococci is approximately 2.3 per cent. The evidence in Part II, series 1, 2 and 3, is that the upper limit of the optimal zone is some place between a

concentration of 18 per cent and 22 per cent carbon dioxide. It is obvious that the optimum concentration is not limited to a definite percentage of carbon dioxide, but may be obtained over a fairly wide zone of carbon dioxide enrichment.

PART IIa. EFFECT OF CARBON DIOXIDE BEYOND THE OPTIMAL RANGE

Valley and Rettger (26) in studying the influence of carbon dioxide on bacteria demonstrated that certain bacteria, such as L. acidophilus, were benefited by carbon dioxide in percentages as high as 25 to 30 per cent. Vigorous and resistant bacteria, such as Bacterium aerogenes and Staphylococcus aureus, grew well in an atmosphere of 95 to 97 per cent carbon dioxide on both plain nutrient agar and highly buffered agar.

Cohen and Fleming (27) demonstrated that primary cultivation of the meningococcus was enhanced by carbon dioxide enrichment. The optimum concentration varied from ten to 30 per cent carbon dioxide, depending upon the strain. With 50 to 75 per cent carbon dioxide the growth was scant.

It was felt of interest to determine the reaction of gonococci in primary growth to carbon dioxide in percentages above the optimum concentration.

In this experiment the chocolate agar employed in the previous work was used. Culture was made, as usual, from gonorrheal discharges of male patients.

The percentages of carbon dioxide tested and other data are supplied in Table 8. The average size of the colonies has been inserted beneath the colony count of four strains.

RESULTS OF PART IIa.

The three cultures of series 1, Table 8, grew over the entire range of carbon dioxide concentrations, from two per cent to 50 per cent. The drop in population above a carbon dioxide concentration of 22 per cent was not abrupt, and, except in the case of culture #3, the growth under 50 per cent carbon dioxide was one-third to one-fourth as great as under the "candle" condition. The average colony size as shown in cultures 1

and 2, changed from a maximum of 1.5 mm. under two per cent carbon dioxide to an average of 0.5 mm. under 40 per cent carbon dioxide. Under a concentration of 50 per cent carbon dioxide the colonies were less than 0.5 mm. in diameter, but distinct. The blackening of pinpoint colonies due to the oxidase reaction was as sharp as that of the larger colonies.

Before dye was placed on the plates, transfers were made from the 40 per cent carbon dioxide plate of culture 1 to fresh chocolate agar. Transfers were also made from the 40 per cent and 50 per cent carbon dioxide plates of culture 2. The organisms grew luxuriantly when incubated under "candle." They were morphologically typical in appearance and fermented dextrose only when grown in single sugars.

Gram stains were made of five colonies from the 1:100 dilution of each condition ("candle," 2 per cent, 10 per cent, 14 per cent carbon dioxide, etc.) of culture 1. The gram negative diplococci grown under the "candle" and two per cent carbon dioxide were typical coffee-bean-shaped organisms, regular in shape. Organisms from the 10 per cent carbon dioxide plates were for the most part regular in shape, but a few bizarre forms were present. These were irregular in shape, frequently triangular, and readily differentiated from the "balloon" forms of gonococci frequently encountered in 48-hour cultures grown aerobically or under low concentration of carbon dioxide. The bizarre forms were present in greater numbers in stains made from plates grown under high concentrations of carbon dioxide. In stains made from the 30, 40, and 50 per cent carbon dioxide plates no regular diplococci could be distinguished.

In Series 2, culture 4 failed to grow above a concentration of 18 per cent carbon dioxide. Cultures 5 and 6 reacted differently to the maximum concentration of carbon dioxide. A sharp drop occurred in the growth of culture 5 above 50 per cent carbon dioxide, while culture 6 grew well under a 60 per cent enrichment. The average colony size of cultures 5 and 6 decreased progressively under increasing concentrations of carbon dioxide.

In Series 3, the maximum concentration was increased to 70 per cent carbon dioxide. None of the cultures of Series 3 produced colonies under the maximum concentration of gas which could be seen with the unaided eye. No growth could be seen under the low-power objective of the microscope against the dark background of the plates. However, when dye was sprayed on the plates minute black specks appeared which simulated the distribution pattern of colonies. Stained preparations were of no aid in determining whether or not organisms were present, for no distinct bizarre or other forms could be distinguished.

Cultures 8 and 9, like Culture 6, apparently were resistant to the effects of 60 per cent carbon dioxide. Cultures 10 and 11 were inhibited.

It would appear a reasonable assumption from the data in Table 8 that gonococci will grow moderately well under high concentrations of carbon dioxide. A few strains in the short series tested grew under a concentration of 60 per cent carbon dioxide. There is an indication that strains of gonococci vary in their resistance to carbon dioxide in high concentrations.

PART III. EFFECT OF GASES OTHER THAN CARBON DIOXIDE ON THE GROWTH OF GONOCOCCI

So far as we have been able to discover, the effect of gases other

than carbon dioxide on the growth of gonococci has been tried only by Rockwell and McKann (8) and McLeod and his associates (11). Reference has been made before to these experiments. It was mentioned that Rockwell and McKann studied the adaptation of gonococci to various gaseous environments after the strains were isolated under reduced oxygen tension. They found such strains grew under pure hydrogen but not under carbon dioxide or oxygen. Strains isolated under partial oxygen tension which would not grow under pure carbon dioxide or pure oxygen could be adapted to do so. McLeod and his colleagues were influenced to try the effect of hydrogen on primary growth of gonococci from the reports of Rockwell and McKann. No advantages were observed from the use of hydrogen.

As final experiments in this study, it was felt desirable to try the effect of hydrogen, carbon monoxide, and nitrogen. Hydrogen was selected for test because the details of its use by McLeod are unknown. Carbon monoxide was included in the experiments since it undoubtedly is formed in small quantity by the burning of a candle in a closed container. Nitrogen was included since it exists in the largest percentage of any gas in air, and its effect on the growth of gonococci has never been reported. The effect of oxygen was not tested because the cultural experiments of many workers have demonstrated the micro-aerophilic requirements of the gonococcus.

In the first experiment the following atmospheric conditions were provided:

(1) Atmospheric air in a closed container. Relative humidity approximately 100 per cent.

(2) Reduced oxygen tension obtained by a partial vacuum equal to nine cm. of mercury on the open manometer. Relative humidity approximately 100 per cent.

(3) Carbon monoxide, approximately 10 per cent. Relative humidity approximately 100 per cent.

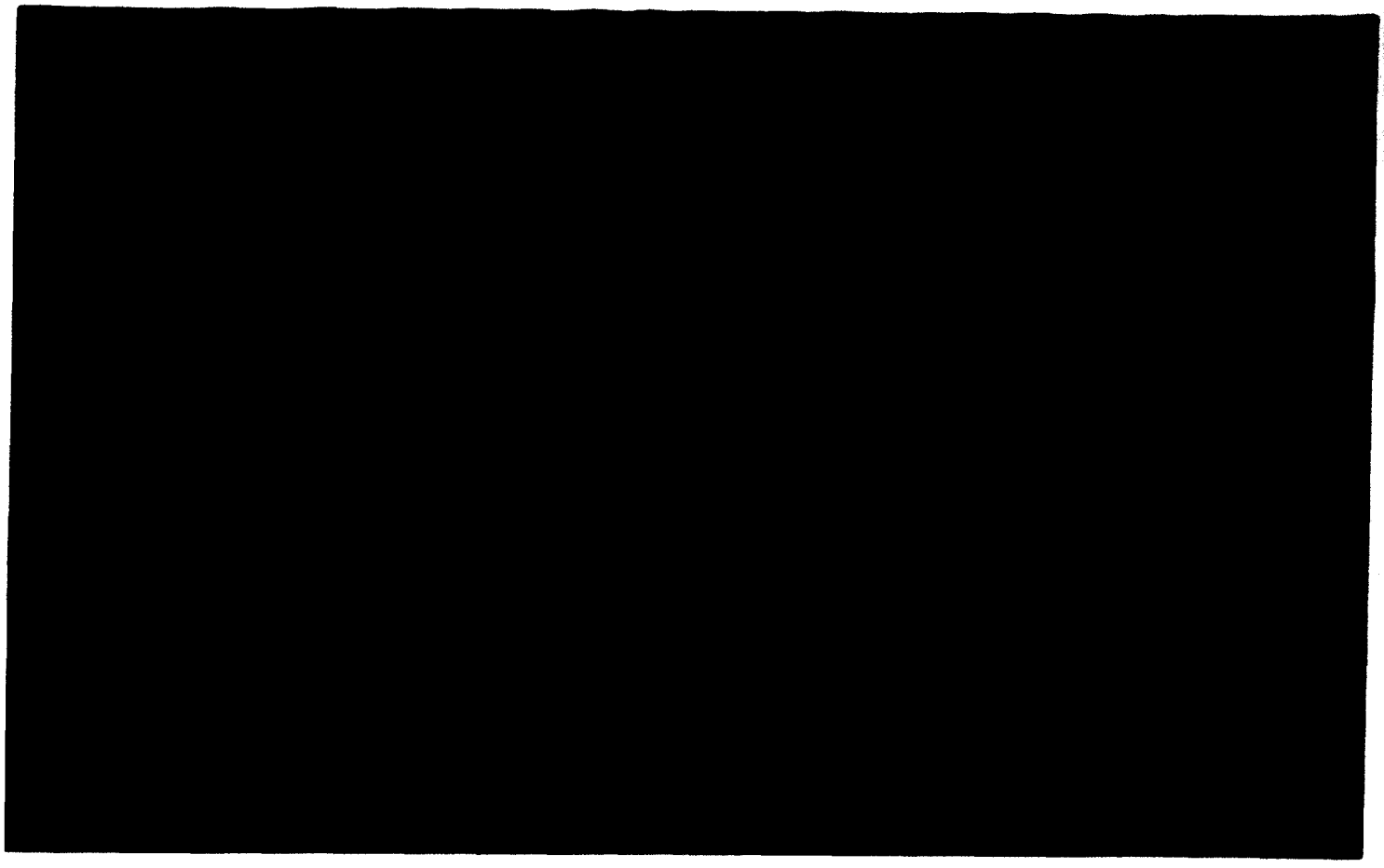
(4) Hydrogen, 10 per cent, supplied by tank. Relative humidity approximately 100 per cent.

(5) Carbon dioxide, 10 per cent, supplied by tank. Relative humidity approximately 100 per cent.

Carbon monoxide was furnished by the action of concentrated sulfuric acid on sodium formate. A simple generator was made by securing the shank of a separatory funnel in the neck of a side-arm flask by means of a one-hole rubber stopper. Acid was placed in the funnel and sodium formate in the flask. The sulfuric acid was allowed to drip onto the sodium formate from the shank of the funnel at a constant rate. Gas from the generator was permitted to flow through rubber tubing connections for twenty minutes before an open U-tube manometer and the culture jar were connected. The carbon monoxide gas replaced ten per cent of air which had been removed by vacuum pump from the culture jar.

Ten per cent concentrations of carbon monoxide and hydrogen were supplied to correspond with a per cent of carbon dioxide from the optimum zone of carbon dioxide stimulation. It was realized that ten per cent carbon monoxide or hydrogen gas might be considerably above or below the possible optimum concentrations of these gases. In this and the following experiments an indication only was sought of inhibitive or growth-promoting properties of carbon monoxide and hydrogen, as well as nitrogen.

Two chocolate agar plates were inoculated directly from collection fluid and two from a dilution tube for each condition.



Thus for each strain and each atmospheric condition, a total of four plates was inoculated. In Table 9, therefore, the significant counts are given in duplicate for nine strains of gonococci.

RESULTS OF SERIES 1

From the counts of Table 9 the atmospheric conditions may be grouped together, or singly, according to the growth stimulation afforded. In ascending order they are:

(1) Aerobic, reduced oxygen tension, hydrogen - poorest growth

(2) Carbon monoxide.....better growth than (1)

(3) Carbon dioxide.....best growth

In Group (1) the counts were very much the same under the three conditions listed, except in the case of Culture 5 which grew under hydrogen only.

Carbon monoxide definitely was superior to the gaseous conditions of group (1) and very nearly on a par with carbon dioxide. Culture #1, however, grew luxuriantly under carbon dioxide and sparsely under carbon monoxide. Culture #7 grew only under carbon dioxide.

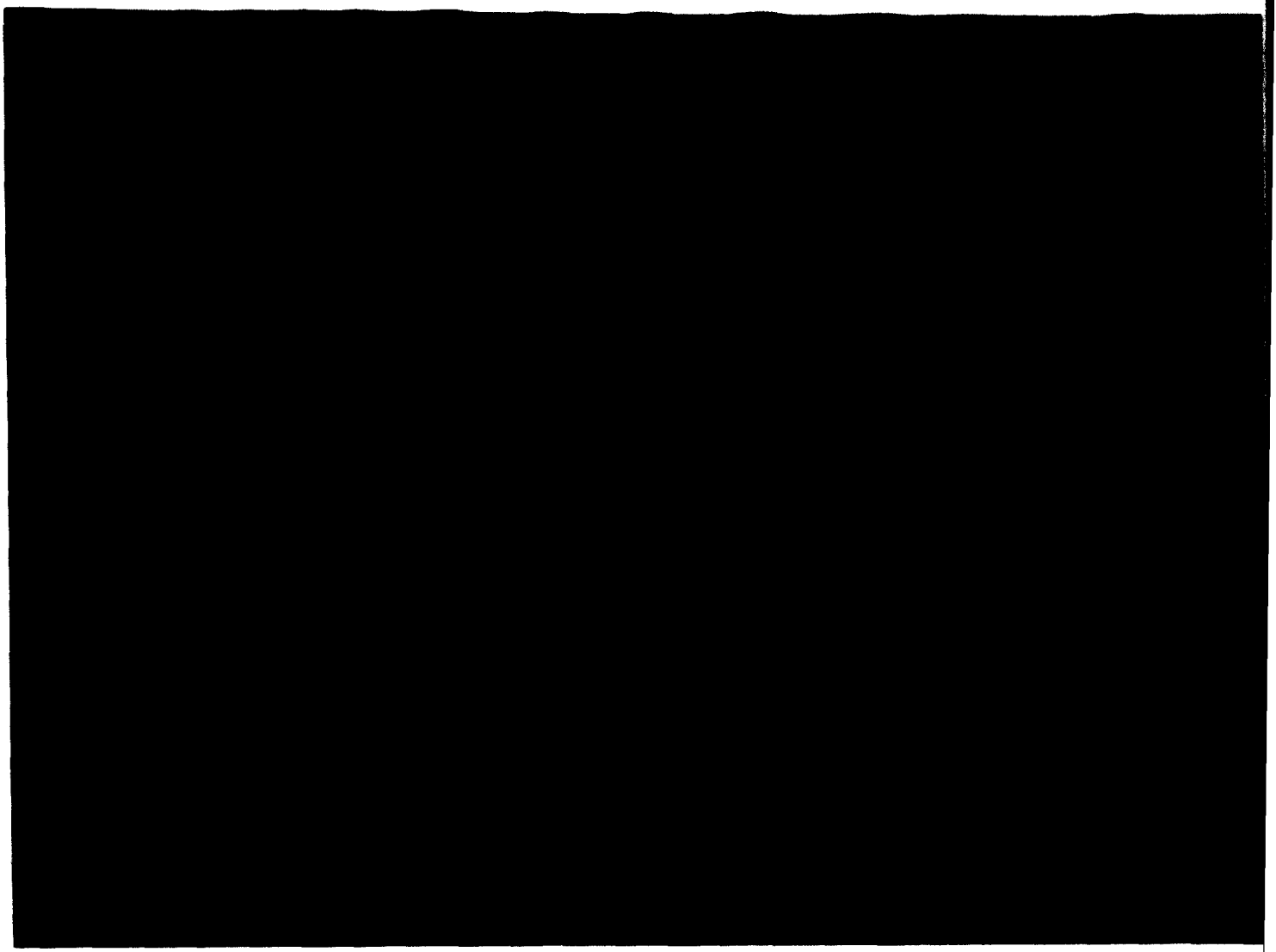
SERIES 2

It appeared from the limited data of Table 9 that carbon monoxide gas provides a stimulus for growth of gonococci greater than that of moist air.

Such evidence could not be accepted, of course, without ruling out the possibility that traces of carbon dioxide in the gas from the generator provided growth stimulation rather than carbon monoxide. A second series of specimens was cultured, therefore, under the conditions of Series 1, with the difference that carbon monoxide gas was bubbled through a column of 40 per cent potassium hydroxide solution containing pyrogallol. A total of sixteen strains of gonococci were cultured. Duplicate platings were made as in Series 1. The plate counts of this series are presented in Table 10.

RESULTS OF SERIES 2

The grouping of atmospheric conditions in Series 2, according to the growth stimulation afforded by them, had to be modified from the order given in Series 1. From the data of Table 10, the relationship was as follows:



(1) Aerobic condition.....poorest growth

(2) Reduced oxygen tension, carbon monoxide, and hydrogen .....better growth than (1)

(3) Carbon dioxide.....best growth

With the exceptions of cultures 7 and 13, the growth of gonococci under reduced oxygen tension, carbon monoxide, and hydrogen was greater than under the aerobic condition. Culture 2 failed to grow aerobically but did grow under the three conditions of Group (2).

Four strains (1, 8, 9, and 14) grew under carbon dioxide but did not grow under other atmospheres. With nearly all cultures, the

population under carbon dioxide was greater than under other conditions.

The surprising growth stimulation caused by carbon monoxide for the dultures of Series 1 was not provided by carbon monoxide bubbled through potassium hydroxide. It is highly probable that carbon dioxide was present in gas produced by removal of water from sodium formate by sulfuric acid.

From this short series there seems little evidence that carbon monoxide and hydrogen in ten per cent concentrations provide any advantages for culture of gonococci that is not furnished by reduced oxygen tension produced by partial vacuum. No deleterious effects could be detected from their use. Since better growth developed under carbon monoxide and hydrogen than under the aerobic condition, it is probable that any value the gases possess in culture of gonococci is in their use for production of a reduced oxygen tension.

PART IIIa. THE EFFECT OF NITROGEN ON GROWTH OF GONOCOCCI

The experiment on the effect of nitrogen did not follow the plan of Part III. The gaseous conditions were somewhat different than in Series 1 and 2 of Part III, since Part IIIa was subordinate to another study.

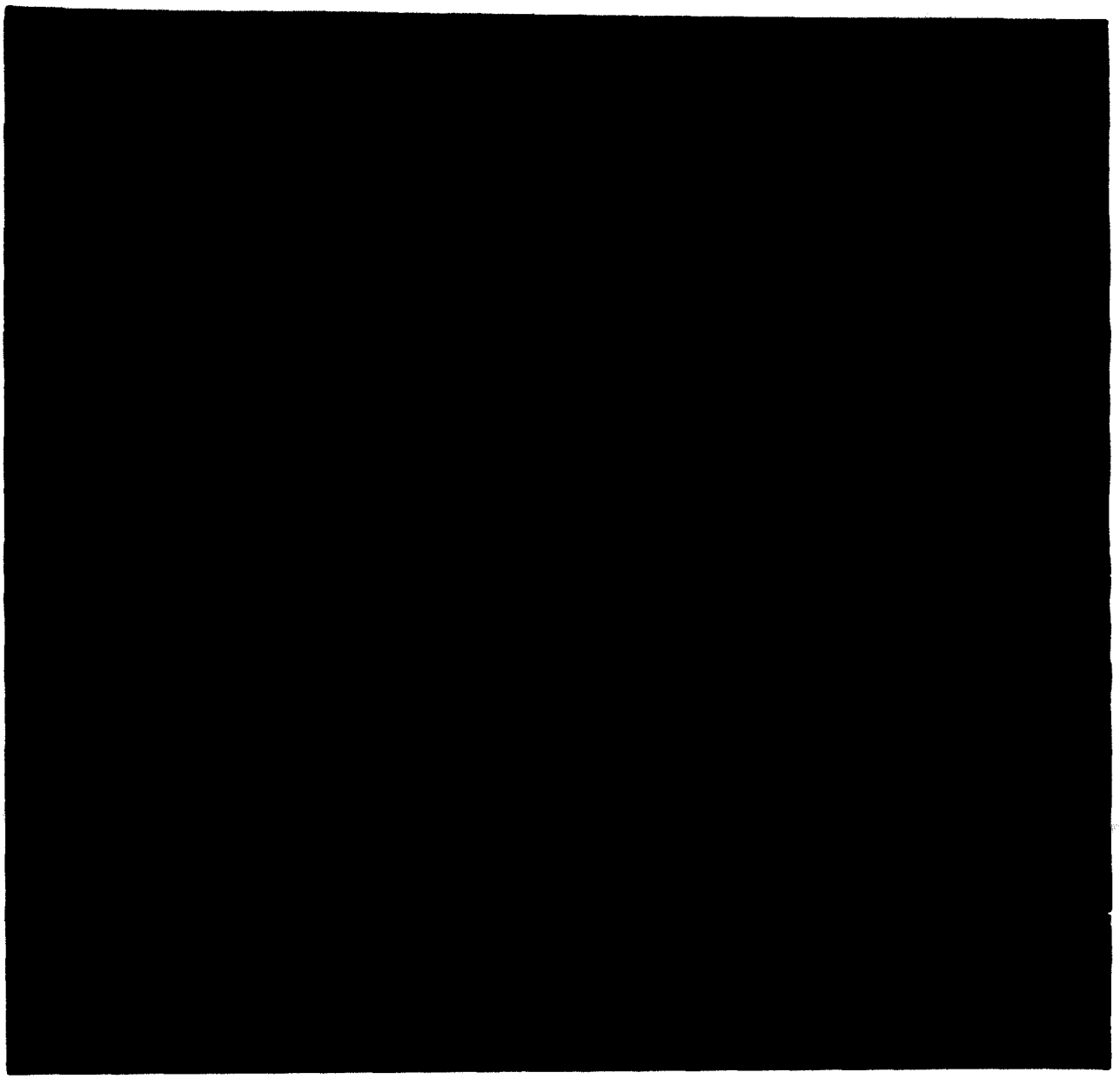
The following atmospheric donditions were provided:

(1) Atmospheric air. Relative humidity 60-70 per cent.

(2) Reduced oxygen tension provided by a partial vacuum of ten cm. of mercury (open manometer). Relative humidity approximately 100 per cent.

(3) Nitrogen 10 per cent, supplied by tank. Relative humidity approximately 100 per cent.

(4) Carbon dioxide 10 per cent, supplied by tank. Relative humidity approximately 100 per cent.



(5) Carbon dioxide supplied by candle. Relative humidity approximately 100 per cent.

Single plates only were inoculated in this experiment for each strain and dilution under the respective atmospheric conditions. The results are presented in Table 11 by the same plate count method used in previous tabulations.

RESULTS OF PART IIIa

It is possible from the counts of Table 11 to group the atmospheric conditions of the experiment in the following order according to the growth stimulation produced:

(1) Aerobic & Nitrogen.....poorest growth

(2) Reduced oxygen tension.....better growth than (1)

(3) Carbon dioxide 10 per cent,"candle"....best growth

It will be noted that despite the differences in moisture content of the aerobic and nitrogen conditions, growth under the two was practically the same, with the exception of culture 5 which grew well under nitrogen and poorly in air.

All cultures grew better under reduced oxygen tension than in air or in air enriched with nitrogen.

The best growth occurred under ten per cent carbon dioxide and carbon dioxide supplied by candle.

No indication is seen from this experiment that nitrogen in ten per cent concentration is beneficial to the primary growth of gonococci. By indirect evidence there is some indication that this amount of gas affords less growth than air saturated with moisture, since growth in an atmosphere only partially moistened afforded growth equal to that of nitrogen.

DISCUSSION

Christiansen and Schoenlein (2) have shown that a candle burned in a closed container provides an environment for the gonococcus quite as favorable as ten per cent carbon dioxide supplied by tank. It is believed by some (28) that the candle supplies approximately 10 per cent carbon dioxide; therefore, its efficacy is the same as a measured amount of gas.

By gas analysis it was shown in this study that approximately 2.3 per cent carbon dioxide is formed by the burning of a candle in a closed container of six liters capacity. This percentage is slightly lower than that given by Nye and Lamb (21) for a container of like size.

Confirmation of the observations of Christiansen and Schoenlein is given in Part I of this thesis where it was shown by culture of specimens from acute cases of gonorrhea that the "candle" environment is the equal of an atmosphere enriched with ten per cent carbon dioxide. Not only were the number of strains of gonococci practically the same but the populations sustained by both atmospheres were found by count to be nearly the same.

Two conditions of partial vacuum providing reduced oxygen tensions equivalent to those produced by ten per cent carbon dioxide enrichment and the "candle" were found to be inferior to carbon dioxide for growth stimulation. Not only in Part I but throughout the study, wherever the conditions of reduced oxygen tension and carbon dioxide were compared, no strain grew under reduced oxygen tension (partial vacuum) that did not grow under carbon dioxide. On the other hand, approximately

25 per cent more strains were isolated under carbon dioxide than under reduced oxygen tension.

In Part I the aerobic condition was found to be inferior to the "candle" and ten per cent carbon dioxide conditions. It is true that the relative humidity of the aerobic environment was less than that provided in the carbon dioxide culture jars. This inequality in moisture content was purposely adjusted, for it was desired that the aerobic condition reproduce the atmosphere and humidity of the average incubator. Where a condition of moisture was provided in the aerobic method equal to that in a carbon dioxide culture jar, as shown in Table 3, the carbon dioxide method was superior, even though the comparison was made with aerobic strains. No strain grew aerobically that did not grow under carbon dioxide in any of the experiments of the thesis.

The effect of moisture on aerobic strains of the gonococcus is strikingly illustrated in Table 3. In some instances growth was 10 or more times greater under a saturated atmosphere than under a relative humidity of 60 to 70 per cent.

A comparison of the efficacy of aerobic and reduced oxygen tension (partial vacuum) environments for growing the gonococcus can scarcely be made from the material in this thesis, since the relative humidity was different when a large number of specimens were cultured in duplicate. In Tables 9 and 10 where comparison is made of aerobic condition and reduced oxygen tension, with the relative humidity the same, there is a suggestion that reduced oxygen tension will provide the better growth.

A plate count was valuable in all the experiments undertaken, but the technique was, without doubt, a necessity in determining the limits of optimal carbon dioxide stimulation. Proper dilution of the original specimen and adequate distribution of inoculum were required to obtain plates suitable for counting. Bent glass rods were the best means found for securing an even spread of organisms.

In Part I it was demonstrated by culture of 68 strains that the gonococcus grows equally well under atmospheres enriched with 2.3 per cent and ten per cent carbon dioxide. In Part II, by culture of 60 strains of gonococci, evidence was presented to show that growth occurs equally well over an even wider zone of carbon dioxide concentrations. The lower limits of this zone are between two and 2.3 per cent and the upper limits from 18 to 22 per cent.

Until the actual nature of carbon dioxide stimulation of the gonococcus is known, it is felt that setting definite limits upon the optimal zone must be done with caution. If, as Valley and Rettger believe (26), the effect of carbon dioxide is beneficial to microorganisms so long as the pH of the medium remains constant, a medium highly buffered, containing non-toxic buffers, might support luxuriant growth of gonococci under high concentrations of carbon dioxide. It is necessary to specify, therefore, that the optimal limits established in this thesis were done with the substrate known as "chocolate" agar, Difco.

It is felt that the establishment of the zone of optimum growth of the gonococcus under carbon dioxide will be of more than theoretical

interest. Medical bacteriologists accept almost slavishly the dictum that primary cultures grow best under ten per cent of the gas. Certain workers in the writer's experience have been discouraged from attempting cultural work in the belief that methods of supplying carbon dioxide are too exacting for the average bacteriologist. While ten per cent, or thereabout, is still the concentration of choice, since it is in the middle of the optimal zone, measurement need not be exact if gas is to be supplied by tank. For routine culture the candle is to be preferred to time-consuming measurement of gas by manometer.

It is not surprising that gonococci are able to grow well, if not luxuriantly, under 30 and even 40 per cent of carbon dioxide. Since the optimal zone is fairly wide, one would expect a gradual increase in the inhibitive effect of the gas above this zone, rather than an abrupt cessation of growth in percentages of gas immediately above 18 to 22 per cent. The data of Table 8 on growth under high concentrations of gas lend indirect support to Part II.

Neither hydrogen, nitrogen, nor carbon monoxide in ten per cent concentrations showed any evidence of stimulating growth of gonococci. As stated in Part III, carbon monoxide was tested to discover if this gas contributed to growth stimulation in a candle jar. The experiment does not entirely answer the question, since the concentration of carbon monoxide in the tests was undoubtedly far greater than the amount present in a candle jar. However, since carbon monoxide is not a normal constituent of air and is not essential to bacterial metabolism, as is carbon dioxide, it is probable that this inert gas does not contribute to growth.

SUMMARY

1. By gas analysis it was established that approximately 2.3 per cent of carbon dioxide is furnished by burning a candle in a closed container of six liters capacity. This percentage is slightly less than that stated by Nye and Lamb.

2. By plate count of strains from acute male cases of gonorrhea, it was found that equal growth of gonococci occurs in a jar in which a candle furnishes carbon dioxide and in a jar whose atmosphere is enriched with ten per cent carbon dioxide. This confirms the observations of Christiansen and Schoenlein.

3. An increase in atmospheric moisture content was found to stimulate growth of aerobic strains of the gonococcus considerably.

4. In all the experiments in which a comparison was made of the efficacy of atmospheric air, reduced oxygen tension produced by partial vacuum, and air reinforced with carbon dioxide, the latter condition was found to produce the best growth of gonococci.

5. Primary strains of the gonococcus were found to grow equally well on "chocolate" agar in a zone of carbon dioxide enrichment with a lower limit between two and 2.3 per cent and an upper limit between 18 and 22 per cent.

6. A moderate growth of gonococci was obtained in percentages of carbon dioxide as high as 40 per cent. The morphology of the organism was considerably changed by growth under high concentrations of this gas.

7. Hydrogen, carbon monoxide, and nitrogen gases in concentrations of 10 per cent in air do not contribute growth stimulation to gonococci.

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