THE EFFECT OF CARBON DIOXIDE IN THE CULTIVATION OF THE MENINGOCOCCUS.

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PLATES 15 AND 16.

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The experiments here reported are the outcome of observations made in applying the recommendation of Cohen and his associates1,2,3 that meningococci be grown at a partial oxygen tension, obtained by substituting carbon dioxide for approximately 10 per cent of the air in a closed container.

The moisture requirement of the meningococcus has been repeatedly emphasized,4 and it seemed important to determine whether Cohen’s results might not be due, in part at least, to the retention of moisture in the partial tension chambers. In our series of carrier examinations two sets of carrier plates were incubated in moist chambers; one set in air, the other in the presence of carbon dioxide, from 2 to 25 per cent by volume. Our uniform experience has been that nasopharyngeal strains of meningococci grow as well, or better, in air saturated with water vapor in a closed chamber as in a similarly saturated atmosphere containing a small percentage of carbon dioxide. The conditions governing the luxuriance of the growth are factors of the humidity of the chamber and the qualities of the medium, and not of the slight differences in oxygen tension resulting from the replacement of a small part of the air with carbon dioxide.

Recently the opportunity presented itself at Camp Zachary Taylor of making similar comparisons with growths of meningococci

from spinal fluids. As a routine approximately 2 cc. of spinal fluid were added to each of six slants of medium. Three tubes of each set were incubated in a water-sealed chamber, in air, while three were incubated in a similar chamber containing 10 per cent carbon dioxide. No significant difference could be observed in the growths under these conditions. The slight differences noted favored the chamber containing air alone. Successive generations were also grown alternately in air and in 10 per cent carbon dioxide, without visible diminution in luxuriance. Two typical strains may be charted by the scheme by which Cohen has illustrated his results. Upstrokes indicate cultures in air, downstrokes cultures in 10 per cent carbon dioxide. One plus sign indicates a single colony, two indicate several colonies, three many, and four a confluent growth.

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Veal infusion agar, with glucose 1 per cent, adjusted to pH 7.4, to which 5 per cent sterile, unheated, citrated horse plasma was added just before tubing. This is a medium highly favorable to the meningococcus.
From these and similar findings the conclusion was reached that the replacement of 10 per cent of air by carbon dioxide has no appreciable effect on the growth of either nasopharyngeal or spinal strains of meningococci, under the conditions of our experiments.

Cohen considers that the effect of carbon dioxide, as he describes it, is not specific for this gas: that it acts merely as a neutral agent, displacing oxygen, and he calls the meningococcus a "microaerophile." A brief consideration of the composition of air, and of the actual displacement of oxygen by 10 per cent carbon dioxide shows that only approximately 2 per cent of oxygen would be displaced and its concentration reduced from 20 per cent to 18 per cent. Such a slight diminution could hardly be expected to produce the marked effects that Cohen reports. Moreover, if the action of carbon dioxide were not specific, results similar to Cohen's should be obtained with any neutral gas, such as nitrogen, which is chemically so inert. The fact that air is composed largely of nitrogen, and that only peculiar species of bacteria are able to utilize nitrogen from the air would seem to make it the most desirable agent for replacing oxygen in testing various oxygen tensions. Therefore nitrogen was used to replace oxygen in the following experiment.

Experiment 1.—January 13, 1919.
Eight plates of veal infusion agar, pH 7.4, with 5 per cent fresh sterile rabbit serum were inoculated with a suspension of a recently isolated spinal strain of normal meningococcus. Two of these plates were placed in each of four large Novy jars containing a little water. In three of the jars concentrations of nitrogen of 10, 25, and 50 per cent, in addition to that already present in the air, were obtained by partial exhaustion of the air and replacement with the gas from a tank. Thus partial oxygen tensions of approximately 18, 15, and 10 per cent were produced in the jars. The fourth jar served as an air control.

After 16 hours incubation the growths of meningococcus were profuse and practically identical in air, in 18 per cent oxygen, and in 15 per cent oxygen. Luxuriant confluent streaks of organisms showed the path of the inoculation. On one of the plates in 10 per cent oxygen the growth was poor, in the other very poor. Only a few small confluent masses of growth showed the site of heaviest inoculation. A plate from each jar is reproduced in Plate 15, Petri Plates 5 to 8.

The meningococcus did not thrive on artificial medium when deprived of half its accustomed oxygen tension. But this experiment does
not indicate that variations of 2 to 5 per cent below normal make any essential difference in its growth. What effect would increased oxygen tension have?

Experiment 2.—January 14, 1919.

Plates similar to those used in Experiment 1 and inoculated as before were divided among the four Novy jars. In a similar manner oxygen was used to replace the exhausted air in percentages of 5, 15, and 25, making the oxygen tensions of the atmospheres in the jars approximately 24, 32, and 40 per cent. The fourth jar again contained air alone.

After 16 hours incubation the growths in all the jars were profuse. Beside the confluent growths where the plates were most heavily inoculated large single colonies of luxuriantly growing organisms appeared. The meningococcus grew as well in 40 per cent oxygen as in air (Plate 15, Petri Plates 1 to 4).

Provided the oxygen tension is sufficient to support its growth, the meningococcus does not seem to be exacting about the concentration of its oxygen supply. These experiments show that the effects of increased carbon dioxide tension, as described by Cohen, are not due to the displacement of oxygen and that the meningococcus is not a "microaerophile." The action of carbon dioxide must be inherent in some specific property of the gas. Carbon dioxide in solution acts as a weak acid and might have a chemical effect on the medium in which meningococci are grown and on the organisms themselves. If serum agar plates, such as are used for isolating the meningococcus, are incubated in a moist atmosphere containing 10 to 30 per cent carbon dioxide, their reaction may be shifted toward the acid end of the pH scale. The following experiment shows this reaction and its effect upon a recently isolated (fourth generation) spinal strain of a normal meningococcus.

Experiment 3.—January 3, 1919.

Four 100 cc. lots of veal infusion agar were adjusted to the following reactions.

<table>
<thead>
<tr>
<th>Lot</th>
<th>Titratable acidity</th>
<th>pH concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.6</td>
<td>7.2</td>
</tr>
<tr>
<td>&quot;</td>
<td>0.8</td>
<td>7.8</td>
</tr>
<tr>
<td>&quot;</td>
<td>0.4</td>
<td>7.9</td>
</tr>
<tr>
<td>&quot;</td>
<td>0.3</td>
<td>8.04</td>
</tr>
</tbody>
</table>

To each lot were added 5 cc. of sterile, unheated rabbit serum, and eight plates were poured. Four plates of each set were heavily inoculated with a
suspension of meningococci from a third generation slant of a spinal strain. Eight plates (one inoculated and one sterile from each lot) were placed in each of four large Novy jars. In three of the jars concentrations of carbon dioxide of 10, 20, and 30 per cent were obtained by partial exhaustion of the air and replacement with the gas from a tank. The fourth jar served as an air control. After 18 hours incubation at 37.5°C. the growths of meningococci were noted and the pH concentrations of the uninoculated plates read with phenolsulfonephthalein and cresol purple as the indicators.

Table I shows the results, and Plate 16 the growths obtained in air, in 10 per cent carbon dioxide, and in 30 per cent carbon dioxide. The growths in 10 and 20 per cent carbon dioxide were practically identical. Several points are to be noted in an analysis of these findings.

The meningococcus does not grow in a medium of a titratable acidity of +0.4 or less when this corresponds to a pH value greater than 7.8 to 8.0 on the Sörensen scale. It may grow luxuriantly on a medium with a titratable acidity of +1.6 when this corresponds to pH 7.2. The fallibility of the old method of titrating media is clearly seen.

The partial saturation of a medium with carbon dioxide from an atmosphere containing 10 to 30 per cent of the gas may increase its hydrogen ion concentration markedly. Almost regardless of the original hydrogen ion concentration, the final reaction seems to represent a state of equilibrium between the carbon dioxide in the medium and in the surrounding air. Thus in the present experiment 10 per cent carbon dioxide brought the more alkaline plates to ap-
proximately pH 7.4 to 7.6, 20 per cent brought them to pH 6.8, and 30 per cent brought them to pH 6.6 to 6.7.

It is evident that such an action of carbon dioxide on the medium might exert either a favorable or an unfavorable effect upon meningococci, according to the original reaction of the medium. A favorable effect is seen in Petri Plates 3 and 4 of all the carbon dioxide series—a medium too alkaline to support growth was brought to a hydrogen ion concentration favorable to the growth of the meningococcus. An unfavorable effect, on the other hand, is seen in Petri Plates 1 and 2 of the carbon dioxide 30 per cent series; the medium was rendered too acid, and the meningococcus grew less well than in air.

Cohen's contention that meningococci which have developed aerobically do not grow well in the presence of carbon dioxide and vice versa is not borne out by this experiment, in which a strain isolated and grown for three generations aerobically grows well in carbon dioxide on a medium in which it cannot grow in air. In connection with the first experiment it may be pointed out that a meningococcus isolated and subcultured in air grew equally well under slightly reduced oxygen tension.

Cohen's reports indicate that this effect of carbon dioxide on the medium has operated favorably in his experiments, and thus the discrepancy between his results and ours may be explained. He and Fleming describe the use of media 0.2 to 0.3 per cent acid to phenolphthalein, under the impression "that the meningococcus will not usually grow when the reaction is over plus 0.5 [per cent] acid to phenolphthalein." In serum agar, 0.2 to 0.3 per cent acid usually corresponds to pH 8.2 to 7.8 on the Sörensen scale, according to the amount of buffer present. Kligler has recently found that the range of growth of the meningococcus in serum dextrose broth is pH 6.1 to 7.8, with the optimum at pH 7.4, and this fact, in conjunction with the effect of carbon dioxide in shifting the reaction toward the acid side, as shown above, suggests that incubation in a partial tension of carbon dioxide has made Cohen's media less unfavorable to the meningococcus, and so, combined with the undoubted aid of

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6 Kligler, I. J., personal communication.
moisture in the closed container, produced the results that he describes.

As for the effect of carbon dioxide on the organisms themselves, a specific action is not so easily demonstrated. That partial tensions up to 30 per cent may not be injurious is shown by the growths on the less acid plates of this series. On the other hand, Shaw-MacKenzie\(^7\) states that carbon dioxide, 22 volumes per cent in Ringer-Locke solution, causes the death of the meningococcus in 20 minutes and suggests that “even the CO\(_2\) normally occurring in the plasma and body fluids may form part of the protective processes of the body.”

**SUMMARY.**

The meningococcus is not a “microaerophile.” It grows equally well in atmospheres containing from 15 to 40 per cent oxygen.

If small amounts of carbon dioxide affect the growth of the meningococcus on an artificial medium it is by changing the reaction of the medium, not by slightly reducing the oxygen tension of the surrounding air.

The fallibility of titrating the total acidity of a medium is again clearly demonstrated. A reaction favorable to the meningococcus cannot be determined from the total titratable acidity but depends solely upon the hydrogen ion concentration of the medium. The optimum for the meningococcus is approximately at pH 7.4.

The value of a moist chamber in the cultivation of the meningococcus is shown by unusually luxuriant growth when other conditions are also favorable.

**Addendum.**

Since the completion of these experiments and the receipt of this paper for publication, St. John (St. John, J. H., *Med. Rec.*, 1919, xcv, 184) has reported his experiments on “Oxygen tension in its relation to the meningococcus.” He concludes that the oxygen tension factor is at least of minor importance in comparison with the effect of moisture in promoting the growth of meningococci. He calls

attention to the fact that the meningococcus is known to be especially sensitive to the degree of moisture and the reaction of the medium, and suggests that "the reaction of the meningococcus medium may be favorably influenced by certain gases evolved by the growing B. subtilis, but this consideration is also minimized by comparison with moisture controls."

At the time that St. John's article appeared there was in press a paper by Frederick L. Gates and Edgar T. H. Tsien on "The effect of moisture on the growth of the meningococcus" which was to have appeared in this number of The Journal of Experimental Medicine. Our findings on the importance of moisture in the cultivation of the meningococcus merely corroborated St. John's, and added nothing but emphasis to his conclusions. Our paper was therefore withdrawn from publication. We feel, however, that the degree of humidity most favorable to the meningococcus is not to be obtained easily unless closed containers are employed.

EXPLANATION OF PLATES.

PLATE 15.

The effect of different oxygen tensions on the growth of the meningococcus.
Petri Plates 1 to 3. 16 hour growths of a spinal strain under increased oxygen tension. Petri Plate 4. A control growth in air.
Petri Plate 5. A control growth in air. Petri Plates 6 to 8. 16 hour growths of a spinal strain under decreased oxygen tension.

PLATE 16.

The effect of carbon dioxide on the growth of the meningococcus, caused by changing the hydrogen ion concentration of the medium.
Petri Plates 1, 1, 1. Original pH 7.2; titratable acidity 1.6 per cent.
Petri Plates 2, 2, 2. Original pH 7.8; titratable acidity 0.8 per cent.
Petri Plates 3, 3, 3. Original pH 7.9; titratable acidity 0.4 per cent.
Petri Plates 4, 4, 4. Original pH 8.4; titratable acidity 0.3 per cent.

In Sets 1 and 2, 30 per cent carbon dioxide increased the hydrogen ion concentration beyond the zone favorable to growth. In Sets 3 and 4, 10 and 30 per cent carbon dioxide brought plates originally too alkaline to support growth into the zone of hydrogen ion concentration favorable to the growth of the meningococcus.
Gates: Cultivation of meningococcus.
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