THE GROWTH OF THE *BACILLUS TUBERCULOSIS* AND OTHER MICRO-ORGANISMS IN DIFFERENT PERCENTAGES OF OXYGEN

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The experiments here described were suggested by the seats of growth of the *Bacillus tuberculosis* in the body corresponding as they do to situations where there is a high pressure of carbon dioxide and a low pressure of oxygen. It was thought from this that the bacillus might either require a certain definite percentage of carbon dioxide in the air, or might be very sensitive to high pressures of oxygen, and only grow, as in the lungs or elsewhere, where the partial pressure of the oxygen is normally much lower than in the atmosphere.

The experiments did not quite justify the theory; but the theory served the most essential purpose of a theory in leading to experiment, and certain of the results have proved in some respects sufficiently interesting to warrant description.

These experiments are still being continued, but as far as they have gone their chief results may be briefly put as follows:—The *Bacillus tuberculosis* either does not grow at all, or grows very badly in the entire absence of oxygen, or in presence of a partial pressure of oxygen amounting to 80 to 90 per cent. of an atmosphere.

A number of other organisms have also been tested, and certain of these, like the *Bacillus tuberculosis*, cease to grow in the higher oxygen percentages, while others appeared to be unaffected by the variations in oxygen. The experiments with the *Bacillus tuberculosis* will first be described, and afterwards those with the other organisms.

Experiment I:—Growth in large tubes with stopcocks, and analyses of the gases in the tubes after growth.

Since carbon dioxide is such a heavy gas, it was thought that under the ordinary condition of culture in test tubes with cotton-wool stoppers

there might be an accumulation of carbon dioxide in the culture tubes in which the bacilli might grow, as in the lung. Accordingly two extra large tubes of about 120 c.c. capacity were made, each with a draw-off tube for analysing the tube gases, closed by a small glass tap near the bottom; and one tube was plugged with cotton-wool in the usual way, while the other was closed above with a rubber cork, through which passed a small glass tube, also closed by a glass tap. Each tube was inoculated on a slant surface of glycerine agar¹ with a strain of Avian tubercle, and the two tubes were incubated alongside of each other for fourteen days at 36° C.

Then the gases in each tube were analysed by drawing samples off into a Hempel gas burette in the usual way. The tube plugged with cotton-wool showed 19·4 per cent. of oxygen and no carbon dioxide; while that closed air-tight by the rubber cork gave 18·7 per cent. of carbon dioxide and no oxygen. Hence the reply of the experiment is that there is no accumulation of carbon dioxide in growing in the ordinary way with wool-stoppered tubes. There was a good growth of the bacillus in both tubes. This appears to be contradictory to the result of some of the later experiments, which show no growth in hermetically sealed tubes in absence of oxygen; but it is to be remembered that in the later experiments ordinary small test tubes were used, in which the supply of oxygen would soon be exhausted, while here, in order to get sufficient gas samples, large tubes of about 120 c.c. capacity were used, and there would be a sufficient amount of oxygen to allow of a good growth before the oxygen became exhausted.

Experiment II.—Effect of high partial pressure of oxygen.

This experiment and some of the later ones were carried out in a small copper autoclave, kept in an incubator at 36 to 37° C., and filled with the desired oxygen mixture, while the controls were grown in the same incubator outside the autoclave but alongside it. The autoclave was used because it was intended to pass on later to oxygen pressures higher than atmospheric, but when it was found that growth was stopped at 80 to 90 per cent. of oxygen at ordinary atmospheric total pressure, the autoclave was not found to be necessary, and hence in later experiments a glass desiccator on a ground base with a mercury seal all round outside the glass junction was used, as subsequently described.

Six tubes containing cultures on glycerine agar of *Bacillus tuberculosis* (Avian) were used for the experiment, four being placed in the autoclave

1. Veal broth agar containing 5 % glycerine, and 2 % peptone, and acidity $\frac{N}{100}$ to phenol phthalein.

with a vessel containing soda lime to absorb any carbon dioxide set free, while the other two were grown in air as controls outside the autoclave in the same incubator. The autoclave was exhausted and then joined up to a gasometer holding oxygen, prepared carefully in the laboratory from potassium permanganate.

The exhaustion and filling up was repeated four times, and at the end the autoclave atmosphere was found by analysis to contain 87·5 per cent. of oxygen. The experiment was begun on June 24th, 1908, and the autoclave was opened and results noted and compared with control on July 8th, 1908.

The following show the percentages of oxygen in the autoclave on different days:—June 24th, 87·5 per cent.; June 26th, 85·7 per cent.; July 1st, 85·1 per cent.; July 4th, 84 per cent.; July 8th, 63·2 per cent. There was always an analysis made for carbon dioxide, but none was found, showing that the soda lime was quite effective. The drop in oxygen in the last analysis indicates the starting of a very slow leak around the rim of the autoclave; but there was never less than sixty-three per cent. of oxygen, and in the earlier part the percentage was nearly ninety.

On opening the autoclave it was found that the four tubes grown inside showed practically no growth at all, while the two controls grown in air showed a moderate growth.

Experiment III.—Growth of Bacillus tuberculosis (Avian) in ordinary small test tubes (a) hermetically sealed, (b) stoppered with rubber corks, and (c) in controls grown in the ordinary fashion with cotton-wool stoppers.

Eight test tubes containing the glycerine agar culture medium were inoculated with Avian tubercle on June 17th, 1908; four of these were hermetically sealed off at the upper end, avoiding any injury to the medium; two were stoppered with ordinary rubber corks; and two were closed with cotton-wool in the usual fashion. The whole eight tubes were then incubated alongside of one another in the same incubator for twenty-one days (till July 8th, 1908), when the condition of growth was noted in each set, and the gases analysed in the hermetically sealed and in the rubber closed tubes respectively.

The two control tubes showed a good growth, the four hermetically sealed tubes showed very slight growth, and the two rubber stoppered tubes showed slightly more growth than the sealed tubes, but much less than the controls.

The glass point of one of the hermetically sealed tubes being broken under water, a negative pressure was shown by an in-rush of water. The

free capacity of the tube was about 40 c.c. and 26·8 c.c. of residual gas was obtained. This contained 0·7 c.c. of carbon dioxide (2·6 per cent.) and no measurable amount of oxygen, so that practically all the residual gas was nitrogen. A similar analysis in one of the rubber stoppered tubes gave 29·6 c.c. of total gas, containing 3 per cent. of carbon dioxide, no oxygen, and the balance nitrogen.

Experiment IV.—Growth of Bacillus tuberculosis (Avian) in high partial pressure of oxygen.

Six tubes of glycerine agar, plugged in the usual fashion with cotton-wool and equally inoculated with Avian tubercle, were taken (July 15th, 1908), and of these four were grown in the autoclave in increased oxygen, while the other two were grown in air alongside in the same incubator. The autoclave after receiving the four tubes, and also an open vessel containing 25 grammes of soda lime, was screwed up, exhausted by a water pump, and allowed to suck in oxygen from a reservoir, the oxygen being made previously, as in all the experiments, from potassium permanganate. The exhaustion and filling was repeated five times, and the final percentage of oxygen in the autoclave was 82·6. The experiment was run for twenty-one days, viz., from July 15th to August 5th, 1908, and analyses of the autoclave atmosphere at intervals gave the following results:—July 18th, 75·5 per cent.; July 22nd, 70·9 per cent.; July 26th, 57·9 per cent.; August 5th, 50·3 per cent.

There is here again a slow leak in the autoclave packing, and an oxygen percentage varying from 82·6 at the beginning to 50·3 at the close. There was no appreciable amount of carbon dioxide present throughout.

Examined at the end the two control tubes show a fair growth, while the four tubes grown in the increased oxygen show practically no growth.

Experiment V.—Growth in stoppered large tubes alongside cotton stoppered similar tubes.

In order to examine more fully the gaseous exchanges in growth in sealed tubes where the growth was finally inhibited, and also to examine the effect of the medium alone upon the air in the sealed tubes, the following experiments were carried out in larger tubes than usual, the capacity of each tube being about 120 c.c. Six culture tubes were taken for the experiment and treated as follows:—

A.—Control. Glycerine agar inoculated with Avian tubercle and plugged with cotton-wool in the usual manner.

B.—Same as A, but plugged air-tight with solid rubber stopper.

C.—Glycerine agar, *not inoculated*, but plugged exactly like B.

D and E.—Same as B, and inoculated, but with a glass tube sealed at outer end inserted through rubber cork so as to be easily broken afterwards in rubber tubing to allow sample of gases to be taken for analysis.

F.—Glycerine agar, *not inoculated*, arranged as in D and E.

The six tubes were grown together in the same incubator from July 23rd till August 21st, 1908 = twenty-nine days.

The final result was as follows:—

<i>Amount of Growth</i>	<i>Analysis of Residual Gases</i>
A. The whole surface of the medium covered with <i>T. B.</i> growth.	Open to air; therefore no analysis.
B. Fair growth, roughly about one-fifth of surface covered	Total volume 83·4 c.c.; no oxygen; carbon dioxide 5·7 per cent.
C. Not inoculated; no growth.	Total volume 84·6; 12·4 per cent. of oxygen; no carbon dioxide.
D. Hardly any growth.	Total volume 74·8; no oxygen; 5·0 per cent. carbon dioxide.
E. Moderate growth; less than B, more than D.	Total volume 82·9; no oxygen; 6·7 per cent. carbon dioxide.
F. No inoculation; no growth.	Total volume 80; 11·5 per cent. oxygen; no carbon dioxide.

This experiment shows that the control grown under atmospheric oxygen gives by far the best growth; that there is an up-take of oxygen by the medium alone but no output of carbon dioxide, about half the available oxygen being so used up in the time; and that in the inoculated and stoppered tubes all the oxygen disappears, but only about one-third of the corresponding amount of carbon dioxide appears.

Experiment VI.—Growth of strains of Bacillus tuberculosis of Avian, Bovine and Human origin, in increased percentage of oxygen.

This experiment, instead of being carried out in the autoclave, was done in a large desiccator or bell-jar, resting on a well-fitting ground glass base, and having a glass tube and well-fitting tap above fixed in a rubber cork in a wide tubulure. In order to keep a quite tight joint below, a mercury seal was arranged all round the base by constructing a circular mound of putty all round about a centimetre high and of about two centimetres greater diameter than the rim of the bell-jar. In this way the slow leak of the autoclave experiments was avoided, and, as shown by the analyses in this and the succeeding experiments, the percentage of oxygen was kept high and very nearly constant throughout the necessarily somewhat prolonged experiments.

Two tubes each were inoculated on glycerine agar with tubercle bacilli of avian, bovine and human origin, and plugged in the ordinary way with cotton-wool. These were placed in the bell-jar above described along with 20 grammes of soda lime in an open flat vessel. One tube of each strain was inoculated and grown in air outside the bell-jar, but close alongside it in the same incubator, to serve as a control.

The experiment was commenced on the 2nd December, 1908, and concluded on the 24th December, 1908 = twenty-two days. The percentage of oxygen was got up at the commencement in the usual way by exhausting and connecting with a reservoir containing permanganate oxygen. The initial value for the oxygen percentage was 77·3 on December 2nd and 76·3 on December 24th, showing that there was no appreciable leak.

Examination of the nine tubes at the later date shows in every case that the growth is stronger and thicker in the controls than in those grown in the oxygen. This is particularly well shown in the human strain, in which one of the two tubes shows hardly any growth and the other a very scanty growth, while there is a much better growth in the control.

The same holds for the bovine growths, but all three tubes are somewhat further advanced.

In the avian the growth is considerable in both oxygen grown tubes and controls, but the control is thicker and better grown.

Experiment VII.—Growth of Bacillus tuberculosis (Human) in increased oxygen percentage.

Three tubes were used of human strain, grown as before on glycerine agar. Two were grown in the bell-jar with increased oxygen, one as control in air outside and close to the bell-jar in the same incubator. Bell-jar exhausted and refilled six times; final oxygen percentage on January 1st, 1909, was 90·5. Experiment continued till January 27th = twenty-six days, when percentage of oxygen was 88·9; no carbon dioxide present.

Examination showed that there had been no growth in the two tubes kept in the oxygen, and a moderate but quite obvious growth on the air grown control tube. As the control had dried somewhat during the twenty-six days of the experiment, in the succeeding experiment this was obviated by placing the control also under a similar bell-jar and in all respects in similar condition to the other set of tubes, except that the growth was made in air instead of in a high percentage of oxygen. But

it may be pointed out in regard to this present experiment, that any drying would militate against growth rather than favour it, yet the control had obviously grown, while the oxygen grown tubes had not grown.

Experiment VIII.—Growth of Bacillus tuberculosis (Human and Bovine) in increased oxygen percentage.

Four culture tubes each, of human and of bovine strains were made, and wool-stoppered in the usual manner.

Two tubes of each strain were placed in the oxygen bell-jar, which was exhausted and refilled in the usual way five times, the final percentage of oxygen obtained being 87.6. Alongside this was placed another similar bell-jar, fitted up in the same way but filled with atmospheric air, and in this likewise two tubes of each strain were placed.

The experiment was commenced on February 14th, 1909, and on February 24th and from that date onward a most marked and increasing difference was observable in the two sets of tubes; those in the oxygen had not appreciably grown, while the surfaces of the air cultures were dotted over with vigorous colonies of growth. The experiment was discontinued on March 3rd, when the percentage of oxygen was found to be 75.

The photographs shown in fig. 1 were made from this experiment.

In the photograph the four upper tubes were those grown in the oxygen, and the four lower tubes were those grown in air. In each series the two tubes on the left-hand side are bovine tubercle, and the two tubes on the right are human tubercle.

In order to test whether the growth of the bacillus was only inhibited by the oxygen, the organisms still remaining alive, or whether they had been permanently destroyed by their stay in the higher oxygen percentage, sub-cultures were made of both bovine and human, from both the tubes in air and those in oxygen. The results showed a good growth in about fourteen days on the sub-cultures from the air grown tubes, while the sub-cultures from the previously oxygen grown tubes showed no growth, but turned brown where there were specks of the inoculated material on the agar. These sub-cultures were, of course, in both cases grown in air. The experiment, therefore, shows that in this case the oxygen had killed the bacteria; but the experiment requires repetition.

Experiment IX.—Growth of Bacillus tuberculosis (Human and Bovine) in increased oxygen percentage.

In this experiment other micro-organisms were cultured alongside the *B. tuberculosis* under the same oxygen and air bell-jars, but the

FIG. 1. TUBERCLE BACILLUS GROWN IN AIR AND IN OXYGEN.

growth of the tubercle bacillus is so slow that the tubes containing the tubercle bacilli had to be kept in the bell-jar during more than one experiment on the other organisms, which grew abundantly in two or three days. For clearness of description the effects on the tubercle bacilli are described separately, but the opening of the desiccator to remove and replace other organisms is noted, and the oxygen percentage at each period in the experiment was usually determined each time the atmosphere was changed.

March 5th, 1909. Two tubes of *Bacillus tuberculosis* (human) and two of *B. tuberculosis* (bovine), which had been inoculated March 2nd, were placed in the oxygen bell-jar containing 90.0 per cent. of oxygen.

Four similar tubes, two of each strain, inoculated at the same time and grown in the interval alongside in the same incubator, were placed in the similar control bell-jar in atmospheric air.

The two sets of tubes show a commencing growth in each case, but slightly advanced.

The two bell-jars placed alongside of each other in the incubator at 6.30 p.m., March 5th, the eight tubes being very similar as to growth.

The two bell-jars were opened on March 9th to remove the tubes with other micro-organisms described in the next experiment (see Expt. X). The growths of the tubercle bacillus had not yet advanced far enough for comparison, so they were replaced and the oxygen bell-jar again charged by six exhaustions and refilling with oxygen from the reservoir.² The oxygen percentage at this second charging was 91.7 per cent., and the tubes were not again disturbed till March 11th, when they were reopened to remove the other organisms grown in tubes alongside. The tubes were again replaced in their respective bell-jars, and the oxygen percentage raised in the oxygen bell-jar to 88.8.

The bell-jars were again opened temporarily and at once restarted on March 12th, March 16th, and finally for examination at the conclusion of the experiment on March 17th, after twelve days' growth in the oxygen, and in the air alongside for the controls.

The oxygen percentage on March 16th was 90.8 per cent.; it was not determined on March 12th or 17th.

All four tubes grown in air showed good growths at the end of the

2. That the failure of the Bacilli to grow was not due to the frequent exhausting of the bell-jar, is shown by the vigorous growth under such conditions of the *B. coli* and *B. typhosus* grown alongside, and also in subsequent experiments.

period; the human and bovine strains in this experiment grew at about the same rate.

None of the four tubes grown in oxygen showed any growth whatever, and the small amount of growth which had occurred in air during the period from March 2nd till March 5th, when they were placed in the higher oxygen percentage, had turned dark brown in colour, the cultures being obviously dead.

Experiment X.—Growth of other organisms—Staphylococcus albus, and Bacillus coli—in increased oxygen percentage.

March 5th, at 6-30 p.m. Two tubes of *Staph. albus* and two tubes of *B. coli* immediately after inoculation were placed in the oxygen bell-jar and grown in 90 per cent. oxygen. As controls, one tube of each organism was grown in the air bell-jar alongside.

March 9th, 2-30 p.m. The two bell-jars opened and growths compared.

The two tubes of *B. coli* grown in the oxygen showed a growth quite equal to that of the tube grown in air. (See fig. 2.)

On the other hand neither of the two tubes of *Staph. albus* grown in the oxygen showed any appreciable growth, while the control tube grown in air had a very good growth, the whole surface of the medium being covered. (See fig. 3.)

The six tubes were at once photographed, and the results are shown in figs. 2 and 3.

Experiment XI.—Growth in increased oxygen percentage of Staphylococcus aureus, Bacillus coli, and Bacillus typhosus.

March 9th till March 11th, 1909. Two tubes of each organism were grown in the oxygen and in the air bell-jars respectively. Growth was commenced on afternoon of March 9th. When examined without opening on March 10th, afternoon, all six control tubes in *air* show good growths; in the *oxygen* the *B. coli* and *B. typhosus* show good growths, while the *Staph. aureus* shows no growth at all.

March 11th. Both bell-jars opened and tubes examined.

All four *B. coli* tubes show full normal growth.

All four *B. typhosus* tubes show full normal growth.

The two *Staph. aureus* grown in the *air* bell-jar showed full normal growth; but the two grown in oxygen showed the merest trace of growth.

Experiment XII.—Growth in increased oxygen percentage of Bacillus pyocyaneus, Vibrio cholerae, Bacillus dysenteriae (Shiga), Bacillus dysenteriae (Flexner), and Staphylococcus citreus.

FIG. 2. GROWTH OF COLON BACILLUS IN AIR AND IN OXYGEN.

FIG. 3. GROWTH OF STAPHYLOCOCCUS ALBUS IN AIR AND IN OXYGEN.

Experiment commenced at 4 p.m., March 11th; growths examined March 12th at 4 p.m.; oxygen percentage = 88.8. Two tubes of each organism grown in air and two in oxygen.

All ten tubes grown in air show a good full growth. Of the tubes grown in oxygen—

Vibrio cholerae shows good growth, as good as control.

B. dysenteriae (Flexner), growth as good as control.

B. pyocyaneus, growth as good as control.

B. dysenteriae (Shiga) shows no growth. (See, however, Expt. XV.)

Staph. citreus. Both the oxygen tubes show an appreciable growth, but much less than controls.

Experiment XIII.—Growth in increased oxygen percentage of *Bacillus diphtheriae*, *Staphylococcus citreus*, *Staphylococcus aureus*, *Staphylococcus albus*, *Bacillus dysenteriae* (Shiga), and *Bacillus dysenteriae* (Flexner).

Examined after twenty-four hours, the two *Staph. citreus* tubes grown in oxygen show a quite perceptible growth, rather more than either *Staph. albus* or *Staph. aureus*, but nothing like the growth of the controls grown in air. The two tubes of *Staph. albus* and *Staph. aureus* respectively from oxygen bell-jar show just the merest trace of growth, practically no growth, while the four air grown tubes show a good growth.

The two *B. dysenteriae* (Shiga) tubes from the oxygen show no growth at all; the two from the air are well grown (see Expt. XV). All four from *B. dysenteriae* (Flexner) tubes, on the other hand, show good growths both in oxygen and in air; there being no appreciable difference.

None of the four *B. diphtheriae* tubes show a good growth, but the oxygen tubes appear to be a little less than the air grown tubes.

Experiment XIV.—Growth in increased oxygen percentage of *Staphylococcus albus*, *Staphylococcus aureus*, *Staphylococcus citreus*, *Bacillus dysenteriae* (Shiga), and *Bacillus dysenteriae* (Kruse).

Four tubes of each of the above organisms were cultivated from March 19th (afternoon) till March 22nd (afternoon). Two of each in air and two in oxygen of 90 per cent.

All the tubes grown in the air showed good growths.

Grown in the oxygen. *B. coli*, good growth, quite as good as controls. *Staph. albus*, fair growth, but not nearly so good as controls. *Staph. aureus*, poor growth, less than *Staph. albus* and not nearly so good as

controls. *Staph. citreus*, fairly good growth, much better than the two previous, and approximately half its own controls. *B. typhosus*, a very good growth, perhaps just a little poorer than the control. *B. dysenteriae* (Shiga), a very poor growth, not to be compared to the control. *B. dysenteriae* (Kruse), poor growth, less than one-third that of control.

In this experiment, which was of longer duration than the previous ones with the more rapidly growing organisms, there was distinctly more growth in the oxygen grown cultures than in the shorter experiments, but, at the same time, the inhibition of certain of the organisms was undoubted. It was also particularly noticeable here, as was more or less obvious throughout the whole series of experiments, that in those cases where the bacteria grew in a suppressed fashion, under the inhibition of the oxygen, that the cultures, instead of forming a more or less uniform mass or smear over the surface of the culture medium, consisted of a number of very marked round colonies heaped up.

In several cases where tubes of staphylococcus which had not appreciably grown while in the oxygen had been left on longer *in air* in the incubator, it was noticed that these recovered and grew fairly well in a rather spotted and heaped up fashion. So that the shorter stay in oxygen does not appear to kill the more rapidly growing organisms in the same manner as the prolonged stay in oxygen appears to kill the tubercle bacillus.

To test the effects of a more prolonged stay in oxygen Experiment XV was carried out.

Experiment XV.—*More prolonged growth in increased oxygen percentage of Bacillus coli, Staphylococcus citreus, Staphylococcus aureus, Staphylococcus albus, Bacillus diphtheriae, Bacillus typhosus, Bacillus dysenteriae (Flexner), Bacillus dysenteriae (Kruse), and Bacillus dysenteriae (Shiga).*

Two tubes of each of these organisms were grown in oxygen of 90·5 per cent. and in air respectively, the experiment being continued from the afternoon of March 25th to that of March 30th.

The following is the comparison of the two sets of growths :—

B. coli, typhosus, diphtheriae show no difference in air or oxygen of any great magnitude, the air tubes perhaps slightly better grown, but difference very slight.

Staph. aureus shows a good growth in air, none in oxygen.

Staph. citreus shows a good growth in air and a considerable growth in oxygen, but in round heaped up masses not occupying the whole surface.

Staph. albus shows a good growth in air, but practically none in oxygen.

B. dysenteriae (Shiga) shows growth in both, but distinctly less in oxygen.

B. dysenteriae (Flexner) shows growth in both, somewhat less in oxygen.

B. dysenteriac (Kruse) was nearly equally grown in both sets of tubes.

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