COMPARATIVE STUDIES ON RESPIRATION.

III. THE EFFECT OF ETHER ON THE RESPIRATION AND GROWTH OF BACILLUS SUBTILIS.

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Although so much stress is laid on the physiological reactions of bacteria in their identification and classification, little investigation (except to distinguish aerobic from anaerobic forms) has been devoted to the fundamental process of respiration in these organisms. In order to throw some light on the nature of this process and to compare the results with those obtained with other plants and with animals, a series of studies upon the respiration of bacteria has been undertaken. The present paper describes the results of some experiments on the effects of ether.

The organism selected for experimentation was *Bacillus subtilis*, isolated from water and inoculated upon agar-agar daily during the month previous to experimentation, so that a pedigreed culture was at hand. Several other organisms were tried; namely, *Bacillus mycoides*, *Staphylococcus pyogenes albus*, *Staphylococcus citreus*, *Bacillus typhosus*; but *Bacillus subtilis* was preferred because it was easier to handle. Bacteria were incubated for 16 to 18 hours at 37°C. and were then washed into a test-tube with a few cc. of tap water containing indicator. A thick emulsion was made by repeatedly drawing the liquid into, and ejecting it from a pipette which was drawn out to a very fine point.

The respiration of the bacteria was tested in boiled tap water. The boiling rendered the water alkaline by driving off the CO₂. To each 2 cc. of solution was added one drop of a 0.01 per cent aqueous solution of phenolsulfonephthalein. The color of the solution was compared with the colors of a set of standard buffer solutions, made according to Sörensen's directions with KH₂PO₄ and Na₂HPO₄, hav-
ing the same concentration of phenolsulfonephthalein as the experimental solution and contained in Pyrex tubes of the same size. The tap water was found to have a pH value of 8.3.

All experiments were done in Pyrex tubes each containing a total of 5 cc. of liquid as follows: Various amounts of tap water plus three standard drops of bacterial emulsion and sufficient saturated solution of ether in tap water to make the following concentrations of ether by volume: 0.037, 0.183, 0.329, 0.438, 0.95, 1.46, 2.9, 4.38, 5.84, and 7.3 per cent.

Each tube, therefore, contained a total volume of liquid amounting to 5 cc. in which the concentration of indicator was the same. The number of bacteria was made as nearly equal as possible in the different tubes by taking a uniform emulsion, mixing it thoroughly before taking the drops from it, and using a standard dropper so as to have the drops of equal size. The emulsion was transferred almost simultaneously to all the tubes. The tubes were then quickly closed (by clean corks boiled in paraffin) with the exclusion of all air and determinations were made by comparison with the standard set of buffer solutions contained in tubes of the same size. In every case there was a control consisting of organisms in tap water without ether, and of ether in tap water without organisms.

To see if phenolsulfonephthalein is toxic to bacteria, several trials were made by placing bacteria in a liquid of known pH value and after respiration had gone on for a definite time adding the indicator, and comparing that result with the color of a control tube to which the indicator was added at the beginning of the experiment. No difference in rate of respiration was observed.\(^1\)

It was also found that ether has no buffer action to interfere with the measurement of the rate of respiration.

That the bacteria under these conditions give off no alkali or acid (other than carbonic) is shown by driving off the CO\(_2\) at the end of the experiment. The solution then returns to the original pH value.

A great deal of variation in cultures of different ages was observed. Those more than 24 hours old have a markedly lower rate

\(^1\) For other details see Paper I of this series (Osterhout, W. J. V., J. Gen. Physiol., 1918, i, 171.)
of respiration than cultures between 18 and 24 hours old. The time required to change the pH value from 8.3 to 8.1 by respiration may be only a few minutes when the culture is young, while as much as 6 hours may be necessary in the case of an older culture. This might be due to the fact that in the older culture the bacteria have gone into the resting stage or have produced spores. In this stage ether has little or no action upon the respiration of the bacteria.

The temperature maintained was from 18-20°C. as this was found to be sufficiently constant. Presumably the respiration of bacteria has the ordinary temperature coefficient of 2. A few experiments were tried at about 1°C. and it was found that exposure to this temperature for 15 minutes had a marked after effect, as little or no respiration was observed for about 6 hours.

In the earlier experiments it was thought desirable to permit respiration to produce a definite change in pH value and then wash out the CO₂ with H₂ or with air free from CO₂. But this was found to injure the bacteria (owing probably to mechanical or chemical disturbances), so that the rate of respiration was greatly diminished. Therefore it was necessary to use separate tubes for the control and for each concentration of ether (instead of finding the normal rate of respiration of a given tube and afterward exposing it to ether). It was possible to do this as a number of readings showed a fairly constant rate of respiration.

The rate is obtained by taking the reciprocal of the time required to produce a given change in pH value. It is expressed as per cent of the normal rate which is always taken as 100 per cent.

Fig. 1 shows a comparison between the respiration of *Bacillus subtilis* in tap water and in four concentrations of ether (by volume). In every case the rate is more rapid at first and becomes slower as the reaction of the medium becomes more acid. The normal curve, indicated by the dotted line, shows a slower rate than any of the other curves. In no case does the indicator show the pH value to be below 6.8 to 6.4.

When higher concentrations are used, the respiration is more rapid during the first few pH intervals and much slower during the last few. In fact, concentrations of ether as high as 1.46 per cent and upward never reach so low a pH value as concentrations below 1.46 per
cent. For example, the curve readily shows that 7.3 per cent ether, although causing a rapid respiration at first, does not produce a lower pH value than 7.9. After allowing these tubes to stand several days, they change about 0.2 pH unit, or reach 7.7 pH, owing probably to the fact that there are a few surviving organisms that are perhaps more resistant than the majority, as later experiments indicate.

Fig. 1. Curves showing the effects of various concentrations of ether on the respiration of B. subtilis. Dotted line, control. Average of two experiments.

The figures expressing the per cent of ether should be corrected by multiplying by 0.73.

Fig. 2 shows more readily the effect of 7.3 per cent ether upon the rate of respiration as compared with the normal rate (dotted line). There is a sudden outpouring of CO₂ followed by cessation of respiration.

Fig. 3 plotted in the same way, shows the effect of lower concentrations of ether upon the rate of respiration as compared with the normal curve (dotted line).

Curve 1 in Fig. 4 shows the rate of respiration in various concentrations of ether during the first interval in which the pH value changes
from 8.3 to 8.1. The rate is taken as the reciprocal of the time required to change the pH value from 8.3 to 8.1. The curve shows a gradual increase in respiration as the concentration of ether increases. The curve rises more rapidly from 2.9 to 5.84 per cent, and very rapidly near 7.3 per cent. In fact, the change in pH value at 7.3 per cent is almost instantaneous.

![Graph showing the effect of 7.3 per cent ether on the rate of respiration of B. subtilis.](https://example.com/graph.png)

**Fig. 2.** Curves showing the effect of 7.3 per cent ether on the rate of respiration of *B. subtilis*. Dotted line, control. The normal rate (which is taken as 100 per cent) corresponds to a change from pH 8.3 to pH 8.1 in 7 minutes. Average of three experiments.

For the sake of comparison, a similar series of observations was made with the same concentrations of ether dissolved in 0.85 per cent NaCl. Curve 2 of Fig. 4 shows increase in respiration with increase in concentration; the curve rises rapidly at first, then more slowly and finally quite steeply. No sudden outpouring of CO₂ is observed in 7.3 per cent ether as is the case in the tap water (Curve 1). This seems to indicate an antagonism between the action of NaCl and ether.
The question arises whether the sudden outpouring of CO₂ in 7.3 per cent ether is due to the sudden production of a great excess of CO₂ or merely to the sudden liberation of CO₂ previously stored up in the cells (either as CO₂ or in the form of carbonates and bicarbon-

![Fig. 3. Curves showing the effect of ether on the rate of respiration of B. subtilis. Dotted line, control. The normal rate (which is taken as 100 per cent) corresponds to a change from pH 8.3 to pH 8.1 in 5 minutes. Average of two experiments. The figures expressing the per cent of ether should be corrected by multiplying by 0.73. It seems more probable that the latter is the case but there is also a sudden increase in O₂ consumption when the bacteria are placed in 7.3 per cent ether, as is shown by using Limulus blood as an indicator.]

2 Osterhout, J. Gen. Physiol., 1918, i, 167.
The great increase in respiration in 7.3 per cent ether in tap water raises the question whether the bacteria were injured. To obtain some light on this question, the contents of each tube were tested (after an exposure to ether lasting 20 minutes) by plating the bacteria on Petri plates and counting the colonies. Two loopfuls of solution from each Pyrex tube were diluted in 10 cc. of sterile water and from these, one loopful was placed in 10 cc. of agar-agar and plates were poured in triplicate. These plates were incubated over night at 37°C. and counted the next day. The results were expressed as per cents of the normal. These per cents are plotted as ordinates in Fig. 5 and the concentrations of ether as abscissae. In performing this experiment all precautions were observed to ensure sterility of the media.

Fig. 4. Curves showing the effect of various concentrations of ether on the rate of respiration of B. subtilis in tap water (Curve 1) and in 0.85 per cent NaCl (Curve 2). The normal rate (which is taken as 100 per cent) corresponds to a change from pH 8.3 to 8.1 in 5 minutes. Average of three experiments.

The figures expressing the per cent of ether should be corrected by multiplying by 0.73.
To show what effect a longer exposure to ether would have upon the bacteria, another set of plates was poured in the same manner as the first set except that the bacteria were allowed to remain in ether for 1 hour. The solutions tried were (1) tap water, (2) saturated solution of ether (about 7.3 per cent in tap water), (3) 0.85 per cent NaCl in tap water, and (4) saturated solution of ether in 0.85 per cent NaCl solution. The following results were obtained, averaging nine trials and counting tap water as 100 per cent.

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Fig. 5 shows that as the concentration of ether increases up to 1.1 per cent there is a decrease in rate of growth, reaching a minimum at about 0.95 per cent. Concentrations of ether from 1.1 to 4 per cent produce increase in growth with a maximum at 2.9 per cent. All higher concentrations of ether produce decrease in growth. These observations show that ether is toxic in low concentrations and in very high concentrations, while intermediate concentrations stimulate growth.

A possible correlation between Fig. 4 and Fig. 5 may be made. In Fig. 4 there is a steeper ascent in the curve up to 1.1 per cent concentration of ether, and this corresponds to the first period of toxicity in Fig. 5. Then there follows a more uniform increase in respiration in Fig. 4 corresponding to an increase in growth in Fig. 5. Above 2.9 per cent ether, Fig. 4 shows the very steep ascent of the curve (or very rapid respiration) and Fig. 5 shows a sudden descent of the curve with a decrease in growth or death of the bacteria.

SUMMARY.

1. In all the concentrations of ether studied (from 0.037 to 7.3 per cent) there is an increase in the rate of respiration of Bacillus subtilis followed by a decrease.

2. In 7.3 per cent ether in tap water there is an extraordinary increase in the output of CO₂ (amounting to 50 times the normal). This does not occur when 0.85 per cent NaCl is added, which indicates antagonism between ether and NaCl.

3. Ether is toxic in low concentrations (0.037 to 1.1 per cent) and high concentrations (3.65 to 7.3 per cent) but in intermediate concentrations (1.1 to 3.65 per cent) stimulates growth.