COMPARATIVE STUDIES ON RESPIRATION.

II. THE EFFECT OF ANESTHETICS AND OTHER SUBSTANCES ON THE RESPIRATION OF ASPERGILLUS NIGER.

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The object of this investigation is to compare the action of anesthetics and some other substances on the respiration of a fungus with similar effects in other groups of organisms.

The fungus which has been used throughout these experiments is Aspergillus niger. This was selected because it grows well in a nutrient solution and forms on the surface of the liquid a compact mass of hyphae, which is easily handled. Penicillium sp. was also tried, but this forms loose tufts of hyphae, which are very hard to manipulate.

The fungus was grown in a nutrient solution of 40 gm. of cane sugar, 2 gm. of KNO₃, 1 gm. of KH₂PO₄, and 0.5 gm. of MgSO₄ in 800 cc. of tap water. This solution has an acid reaction, a fact which may have been beneficial in preventing the growth of bacteria. The cultures were examined at various times for bacteria but at no time were any found. The fungus was grown from spores in 100 cc. Erlenmeyer flasks, in about 30 cc. of sterilized nutrient solution. They were kept slightly above room temperature. Spores were usually formed in 3 or 4 days after inoculation, so it was found best to use the cultures when 2 days old. The respiration was at its maximum at this time. If older cultures were taken respiration was not so great.

The indicator method was used for determining the amount of CO₂ given off. For buffer solutions borax and boric acid solutions were used in various mixtures. A table for making up buffers of

any desired pH value by means of borates and boric acid has been published by Palitzsch and later republished by Osterhout and Haas.

In the experiments 10 cc. of tap water were used in each tube to which five drops of a 0.01 per cent solution of phenolsulfonephthalein were added as indicator. That this indicator was not toxic to the fungus was proved by sowing spores in a nutrient solution containing the same concentration of indicator as used in the experiments. A control with the same amount of spores was inoculated at the same time and grown under similar conditions. The two cultures matured at the same time and behaved similarly when treated with anesthetic.

Respiration was measured in the nutrient solution and in sugar solutions in preliminary experiments, but as the results were the same as when tap water was used, the nutrient solution was replaced by tap water, which was easier to handle. The tap water was very near the neutral point and was brought to the desired alkalinity by boiling off some of the dissolved CO₂ or by washing out some of the CO₂ by means of a current of air free from CO₂.

When an experiment was started, some of the fungus was thoroughly rinsed in tap water to wash off the nutrient solution. Any adhering bubbles of gas enclosed in the interwoven hyphae had to be got rid of before the readings were begun, either by gently squeezing the material between the fingers or by a continued shaking in several changes of water. The mass of hyphae was then separated into pieces small enough to be shaken about easily in the tube. They were then put into a Pyrex glass tube which was closed at one end, while at the other was attached a paraffined rubber tube about 3 inches long. Before putting the fungus in the tube, 10 cc. of tap water (plus five drops of indicator) had been poured in. When the fungus had been put in, the rubber tube was closed with a spring clamp, in such a way as to enclose a small bubble of air, to act as a stirrer. In all experiments this bubble was made as nearly the same size as possible. The tube was now shaken until any gas that may have adhered to the material was detached. The color of the experi-

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The mental tube was then matched with that of a buffer tube of the same size (and having the same concentration of indicator), using a constant source of light ("Daylight" lamp). The alkalinity of the water was brought (by the means mentioned above) to a point a little above pH 7.60; after adding the material it was allowed to stand until it fell to pH 7.60, which was taken as the starting point in all experiments. The time was noted and the tube put aside for a minute or two, when it was gently shaken to distribute the CO₂ throughout the solution. The tube was again matched, this time with another buffer tube having a pH value of 7.25. If the experimental tube had not yet reached this, it was repeatedly examined at very short intervals until the two matched. The change in pH was from 7.60 to 7.25, which was the standard unit of measurement in all cases.

In starting an experiment the time required for this change was noted and the material was rejected unless this time was practically constant for at least three periods before any anesthetic was added. The time varied with the amount of material used, but was made as near to 3 minutes as possible in each experiment. When the endpoint was reached, the material was taken out and rinsed in tap water before starting a new measurement with a new solution.

When a practically constant rate of respiration had been obtained, the material was placed in a solution of the desired concentration of the anesthetic. After the addition of the anesthetic the respiration was measured in the same manner as before. The experiments with anesthetics lasted from half an hour to 2 hours.

On account of the short periods used it was found impossible to carry on a control simultaneously with the experiment. Therefore a large number of control experiments were made from a number of cultures and at different times during the period of investigation. These control experiments agreed very closely, so they were averaged to make up a control curve, which has been used in all figures. As will be noticed from the figures, the control shows a gradual decrease in rate of respiration. This is probably due to a decrease in oxidizable

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4 For other details see the introductory article by Osterhout, W. J. V., J. Gen. Physiol., 1918, i, 171.
material within the cells, as this decrease did not occur when a 3 per cent sugar solution was used in place of tap water.

All the experiments were made at room temperature (18–20°C.).

In regard to the accuracy of the results it may be stated that in no case did the probable error exceed 2.4 per cent of the mean.

The measurement of respiration at frequent intervals, as here practised, has great advantages. Methods heretofore employed in measuring respiration have usually necessitated the use of long periods. It will be noticed readily from curves here presented that if periods of an hour's duration had been used no increase would have been found except in one or two cases, because the increase, though distinct enough, lasts but a short time (in many experiments only from 5 to 10 minutes). This increase is followed by a decrease, and if the total amount of CO₂ given off in 1 hour were measured, the large output for a short time would be more than counterbalanced by the longer period of small output, so that the total would be below normal, and we should record a decrease instead of an initial increase followed by a decrease. In the writer's opinion there is no doubt that this is what has happened in many cases, where only a falling off of respiration has been reported. Schroeder got a decrease in respiration of *Aspergillus niger* when he used 6 and 7 per cent ether. In the present investigation 3.65 and 7.3 per cent ether both gave a decided increase (except in a few cases), but this was followed by a drop, and if 1 or 2 hour periods, as Schroeder used, had been employed, it is very likely that only a decrease would have been noticed. Kosinski as the result of experiments with *Aspergillus niger* reports an increase in CO₂ output with 0.5 per cent ether and a decrease with 5 and 7 per cent; with 0.2 per cent cocaine and 0.02 per cent strychnine nitrate an increase was observed.

The first experiments were made with formaldehyde. A few experiments were conducted at first with 0.2 per cent (by volume), but as this concentration gave a very small change, 0.4 per cent was used. This stronger concentration gave a very large increase followed by an abrupt decrease. The results are shown in Fig. 1. Six experiments

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were made with this concentration. Their average happens to correspond almost exactly with one experiment, and this experiment

Fig. 1. Curve showing the effect of 0.4 per cent formaldehyde on the respiration of *Aspergillus niger*. The shaded portions represent the periods during which respiration was measured. The unshaded portions represent the intervening time during which no measurement was made (e.g., time spent in changing the material from one solution to another). The horizontal part of the curve shows the normal respiration; the remainder the respiration in 0.4 per cent formaldehyde. The dotted line represents the respiration of a control in tap water. Time is reckoned from the beginning of the exposure to formaldehyde. The normal rate (which is taken as 100 per cent) corresponds to a change from pH 7.60 to pH 7.25 in 2.75 minutes.

was used instead of the average in drawing the curve, as will be seen in Fig. 1. The periods during which the measurements were made are indicated by shading. The rate which is obtained for each period
is an average for that period, and if the rate is changing, it is evident that this average rate will in general occur somewhere near the middle of the period. Hence in drawing the curve the points are taken in the middle of each period. The other curves are made in the same way but the periods are not indicated.

After exposure to the reagent has begun, the intervals between periods of measurement are included in the total time of exposure to the reagent, because even when the material is momentarily lifted out, it remains covered by a film of the reagent. The relative rate of respiration for each period is obtained by dividing the rate for that period by the normal rate. This applies to all the subsequent work.

The main part of the work was done with ether. Various concentrations between 0.37 and 1.46 per cent (by volume) were tried in the beginning but as these had little or no effect, higher concentrations were employed. A slight increase of respiration, which lasted for some time, was obtained with 1.46 per cent ether. With 3.65 per cent three distinct types of results were obtained. The first and most common type was a sharp rise in respiration in the first period, followed by a gradual decrease (Fig. 2, Curve A), so that at the end of half an hour the rate was slightly below normal. From this point on the rate decreased more slowly, reaching about 60 per cent of the initial rate at the end of 80 minutes. A second type gave a curve with a flattened top having the maximum rate of respiration in the third or fourth period. Only very few experiments showed this type. The third type never gave a rise, but always a slow decrease from the beginning (Fig. 2, Curve B).

These three types were constant in the sense that each culture always gave only one result, no matter how many experiments were made. Though the cultures were grown under identical conditions, yet this difference in behavior was always noted.

A saturated solution of ether was also tried. As this is approximately 7.3 per cent by volume, the latter designation has been used for the sake of convenience. With 7.3 per cent only one type of result was noticed, even when the same cultures were used with which different results had been obtained with 3.65 per cent. A very pronounced increase was noticed in the first period followed by an almost equally
rapid decrease, so that in from 6 to 10 minutes the rate was back at normal. At the end of an hour respiration was nearly at a standstill (Fig. 2, Curve C).

**Fig. 2.** Curves showing the effect of ether on the respiration of *Aspergillus niger*. Curve A, in 3.65 per cent ether; Curve B, a different culture in 3.65 per cent ether; Curve C, in 7.3 per cent ether. The horizontal part of the curves shows respiration in tap water before exposure to ether. Dotted line shows respiration of a control in tap water. The normal rate (which is taken as 100 per cent) corresponds to a change from pH 7.60 to pH 7.25 in 3.25 minutes for Curve A and in 3 minutes for Curves B and C. Curve A represents the average of four experiments; Curves B and C, the average of five experiments. Probable error, less than 2.4 per cent of the mean.

The results with ether are shown in a different manner in Fig. 3. A number of experiments on recovery were conducted with 7.3 per cent ether. The result of these experiments was, that if respiration
had risen and fallen again to normal and the material was then taken out of the ether solution and put in a nutrient solution, it was found on observing it 2 to 3 hours later that respiration was practically normal (Fig. 4, Curve B). If on the other hand the material was allowed to remain in the ether solution until the rate had fallen to about 60 per cent of the normal and then placed in nutrient solution recovery to normal was not obtained. From this it would seem that only the increase in respiration is reversible and that when a decrease takes place an irreversible reaction involving injury is going on.

A 20 per cent solution (by volume) of acetone was also employed. A very large increase took place, with the maximum during the second period (Fig. 5, Curve B). This concentration of acetone was very much less toxic than 7.3 per cent ether.

Alkaloids usually have a special effect. For this reason it was thought best to try caffeine as a representative of this group. Low
concentrations, such as Haas's reports using, had no effect whatsoever. The lowest concentration that showed any effect was 0.5 per cent (Fig. 5, Curve A). This gave a decrease in respiration from the beginning. In the first two periods there was a decrease of nearly 20 per cent below normal; then followed several periods with hardly any decrease at all. Several experiments with a saturated solution of caffeine were also performed. An initial increase amounting to about 15 per cent above normal was noticed (Fig. 5, Curve C). This was followed by a gradual decrease till a rate of 60 per cent of the normal was reached, when the rate of respiration became stationary and remained so till the end of the experiment (over half an hour).

Fig. 4. Curve B shows the respiration of Aspergillus niger, first for 20 minutes in tap water (horizontal unbroken line), then for 9.5 minutes in 7.3 per cent ether, then for 130 minutes (interval shortened in figure to save space and denoted by dotted lines) in nutrient solution, and finally for 36 minutes in tap water (unbroken line). Curve A shows the respiration of a control placed in tap water for 30 minutes, then for 130 minutes in nutrient solution (dotted line), then for 34 minutes in tap water (unbroken line). The normal rate (which is taken as 100 per cent) corresponds to a change from pH 7.60 to pH 7.25 in 3 minutes. Average of three experiments. Probable error less than 2.4 per cent of the mean.

7 Haas, Science, 1917, xlvi, 462.
Fig. 5. Curve B shows the respiration of *Aspergillus niger*, first for 20 minutes in tap water (horizontal part of curve), then in 20 per cent acetone. Curve A shows the respiration, first in tap water (horizontal part of curve), then in 0.5 per cent solution of caffeine. Curve C shows respiration, first in tap water (horizontal part of curve), then in saturated solution of caffeine. The dotted line shows the respiration of a control in tap water.

The normal rate (which is taken as 100 per cent) corresponds to a change from pH 7.60 to pH 7.25 in 2.75 minutes for Curve A; in 3.25 minutes for Curve B, and in 3 minutes for Curve C.

Curve A represents the average of seven, Curve B of four, and Curve C of two experiments. Probable error less than 2.4 per cent of the mean.
The action of caffeine seems to be very much slower and less pronounced than that of the other substances tried.

The absence of any noticeable buffer action in any of the solutions was determined by means of an apparatus which has been described by Osterhout. A measured amount of CO₂ is introduced into a certain amount of solution with indicator and the change in pH is noted. This is compared with the change caused by an equal amount of CO₂ in the same amount of tap water.

The above results agree in the main with those obtained by Haas on _Laminaria_. Usually stronger solutions were needed to cause any effect on the respiration of _Aspergillus niger_, and the changes were not so great. With all reagents tried there was an increase, though with some concentrations only a decrease was noted.

**SUMMARY.**

1. In concentrations which are high enough to produce any effect, formaldehyde, ether, and acetone cause an increase, followed by a decrease, in the rate of respiration.

2. 3.65 per cent ether, which causes an increase with certain cultures, produces only a decrease with others.

3. The reaction producing an increase in the respiration with 7.3 per cent ether is a reversible process, while the reaction producing the decrease is not reversible.

4. 0.5 per cent caffeine produces only a decrease in respiration while a saturated solution causes an increase, which is followed by a decrease.

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*Osterhout, J. Biol. Chem., 1918, xxxv, 237.*