COMPARATIVE STUDIES ON RESPIRATION.

XI. THE EFFECT OF HYDROGEN ION CONCENTRATION ON THE RESPIRATION OF PENICILLIUM CHRYSOGENUM.

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The effect of hydrogen ion concentration on respiration has received very little attention. Warburg measured the amount of oxygen consumed by fertilized eggs of Strongylocentrotus lividus, when they were in a balanced solution of NaCl, KCl, and CaCl₂. He found that on raising the hydrogen ion concentration from pH 8 (which is that of sea water) to pH 6 the oxygen consumption was lowered to nearly one-third, while on lowering the hydrogen ion concentration to pH 11 the oxygen consumption was more than doubled. Loeb and Wasteneys repeated Warburg's experiments, using Arbacia punctulata. They found that if oxygen consumption at an OH ion concentration of 10⁻⁷ (pH 7) is taken as 1.00, at a concentration of 10⁻⁴ (pH 10) it was 1.17 and at a concentration of 8.4 × 10⁻⁴ (pH 10.92) it was 2.74. Since similar concentrations of NaOH and NH₄OH produced similar effects it appeared doubtful whether the result was to be attributed to the concentration of hydroxyl ions only.

Thunberg, studying the effect of H ions on surviving frog muscles, found that with concentrations of 0.005 M HCl (about pH 2.3) the production of CO₂ decreased to 82.7 per cent of the normal, 0.02 M HCl (about pH 1.7) decreased it to 44.6 per cent while with 0.05 M HCl (about pH 1.2) there was a decrease to 24 per cent of the normal. He found the same concentrations of NaOH less toxic; 0.005

1 Warburg, O., Z. physiol. Chem., 1910, lxvi, 305.
2 Loeb, J., and Wasteneys, H., Biochem. Z., 1911, xxxvii, 410.
m (about pH 11.7) decreased the production of CO₂ to 88.8 per cent, 0.02 m (about pH 12.3) decreased it to 71.3 per cent. He found Ca(OH)₂ more toxic than HCl at equal concentrations. He explains this by supposing that calcium precipitates out phosphates in the tissue. Oxygen consumption was decreased to about the same degree that the production of CO₂ was decreased. Thunberg also used a large number of organic acids and found that most of them had only a slight effect in concentrations from 0.01 to 0.2 m. A few, like formic, α-oxybutyric, and pyroracemic acids, decreased the production of CO₂ from 40 per cent to 50 per cent at the greatest concentration. On the other hand acids like citric, fumaric, and malic increased the production of CO₂ in proportion to the increase in concentration of acid.

Some work has been done on the effect of H ions on the fermentation by yeast. In most cases, however, the pH of the solutions was not measured. Solutions of 0.1 N HCl and HNO₃ seem to inhibit fermentation entirely. Hägglund determined the pH of his solutions and found that the optimum hydrogen ion concentration for yeast fermentation (using lactic acid, HCl, and H₂SO₄) was $7 \times 10^{-4}$ (pH 3.16). His criterion of fermentation was the production of CO₂. Lösers found that the pH of the solution, after fermentation had been in progress for several days, always was from 2.51 to 2.73, though at the beginning it may have been neutral.

The extent to which oxidase enzymes are concerned in respiration is at present unknown, but it is important to note that they are inhibited by acids. Bertrand, as well as Abderhalden and Guggenheim, and later Wolff reported inhibition of oxidase reaction by acids. According to Bunzell the oxidation of hydrochinone by laccase (from alfalfa) increases with decrease of H ions from $5.6 \times 10^{-8}$ to $7 \times 10^{-8}$ (pH 7.26 to pH 8.16). Reed found that the

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5 Hägglund, E., Biochem. Z., 1915, lxix, 190.
7 Bertrand, G., Compt. rend. Acad., 1907, cxlv, 340.
oxidase in potato and apple juice was inhibited, when the solution had had hydrogen ion concentrations of \(5.5 \times 10^{-4}\) (pH 3.26) and \(7.4 \times 10^{-4}\) (pH 3.15) respectively, and that in both cases the optimum is near neutrality. Bunzel\(^{12}\) found that potato juice oxidase was inhibited by pH 3.55 to pH 3.70, and that the activity increased as the pH number increased up to 7. The oxidase of the tulip tree was inhibited at pH 2.30 to pH 2.80, and increased in activity up to pH 6. He also found that the oxidase of magnolia was inhibited at pH 2.45 to pH 3.05 and increased with increasing pH value up to pH 5.70.

In the experiments to be described *Penicillium chrysogenum* was used. This plant was chosen because under the conditions of these experiments it does not produce sufficient alkali or acid (other than carbonic) to interfere with the results. Another reason which made it very good material to work with was its hardiness (it was originally found growing on strong formalin solutions). *Aspergillus niger*, which has been used by the writer in previous work on respiration,\(^{13}\) produces a small amount of a non-volatile acid, for which reason it was not well suited to the method employed in the present investigation.

The way in which the fungus was grown, as well as the procedure employed in measuring the production of CO\(_2\), and the method employed in calculating the rate are described in a previous paper by the writer.\(^{13}\)

The pH of the solution in which the fungus was placed to determine the effect of hydrogen ion concentration was determined by means of indicators, using buffer solutions for comparison. For determining the concentrations around pH 1 to 4 tropeolin OO and methyl red were used, with buffers containing HCl and sodium citrate.\(^{14}\) For the concentrations at pH 7 to 9 phenolsulfonephthalein and phenolphthalein were employed and borate buffers were used for comparison.

The normal respiration was measured in 0.5 per cent dextrose solution in distilled water. To a similar solution sufficient acid or alkali was added to get the desired hydrogen ion concentration.

NaOH was employed for the alkaline solutions. For the acid solutions H$_3$PO$_4$ and H$_2$SO$_4$ were used; to get rid of any volatile acid which might be present the acid was boiled for some time before using.

For preliminary work both H$_3$PO$_4$ and H$_2$SO$_4$ were employed, but as both of these acids gave practically the same results, when the H ions were present in the same concentration, the use of H$_3$PO$_4$ was discontinued. All the results here described were obtained by the use of H$_2$SO$_4$.

In the experiments where the pH was less than 7 the apparatus described by Osterhout$^{15}$ was employed. For the alkaline solutions this method could not be used, as the CO$_2$ neutralized the NaOH, and therefore the method described by Haas$^{16}$ was employed. The method adopted here was to determine the time required to produce a given amount of CO$_2$ in normal respiration at pH 7.30 and then change the pH and determine the time required to produce the same amount of CO$_2$.

This necessitated a determination of the change in pH produced by the same amount of CO$_2$ when added to neutral or alkaline solutions. This was accomplished in the following manner. Into a glass tube 10 cc. of a solution having a pH of 7.30 were put, and into a second tube 10 cc. of an alkaline solution; both solutions contained indicators. As soon as the tubes were filled they were stoppered and compared with standard buffers to get the exact pH. Into each tube one drop of the same solution of CO$_2$ in water was simultaneously introduced. The tubes were shaken and comparisons with standard buffers were again made in order to measure the change in pH. This procedure was repeated several times and the average of the changes in each solution taken as the change to be produced by the fungus. If for example the average of these changes was found to be from pH 7.30 to 7.10 in one solution, and from pH 8.92 to 8.68 in the other, standard buffers were made up for these values. Then the normal rate of respiration was determined by finding how long it took the fungus to change the pH from 7.30 to 7.10. Unless the rate remained constant for at least 20 minutes the material was rejected. (It had been found by experiment that after the respira-

$^{15}$ Osterhout, W. J. V., J. Gen. Physiol., 1918-19, i, 17.
tion remained constant for 20 minutes it would keep this rate for hours provided food was supplied.) After a constant rate had been established, the material was transferred to the alkaline solution and the rate again determined by noting the time required to produce an equal amount of CO₂; i.e., to change from pH 8.92 to 8.68.

The pH of the nutrient solution both before the spores were sown and after the mycelium had grown large enough to be used was found to be about 4. In no case was there any noticeable change during the growth. A few cultures were grown in a nutrient solution having a pH of 6 to 7. These cultures showed no difference in their behavior from those grown in a more acid medium. The medium on which the fungus was originally growing had a pH of 6 to 7. In all the experiments there was some change in the pH value of the solution containing the fungus. The average pH during the experiment is therefore taken in all cases as the pH of the experiment.

In the following account the rate of respiration at neutrality is arbitrarily designated as normal.

The preliminary work was started with solutions having a pH of about 4. With this hydrogen ion concentration there was no apparent effect. Next a solution of pH 2 was tried. Contrary to what was expected this concentration caused a considerable rise in the first half-hour followed by a fall below normal during the second half-hour.

After the preliminary experiments had given this result more careful experiments were made. The first concentration to be used was pH 1.35. This gave a rise of 20 per cent during the first 20 minutes, which was followed by a fairly rapid fall below normal. The second concentration to be used was pH 1.95. The rise in this case was more gradual than in the preceding one and the fall was also much more gradual and did not fall nearly so much. A few experiments were also made with pH 1.10. These were similar to those with pH 1.35 except that the decrease was sharper and greater. Solutions of pH 1.70 gave results very much like those with pH 1.95.

A large number of experiments were made with pH 2.65. These results were rather variable. With only a few exceptions the rate of respiration was always at or above normal during the entire experiment. The experiments that gave the most divergent results were rejected, and from the remainder an average curve was constructed, which for 80 minutes did not fall below normal (Figs. 1 and 2).
Fig. 1. Respiration of *Penicillium chrysogenum* expressed as per cent of the normal (at pH 7). The material was put in the test solution at the point marked 0 on the abscissa and the line at the left of this point represents the normal respiration. Curve A represents the respiration in a solution having a pH of 7 (normal); Curve B in pH 1.10; Curve C in pH 1.95; Curve D in pH 2.65; and Curve E in pH 8.80. The normal rate represents a change in the indicator tube from pH 7.94 to 7.52 in from 2 to 3 minutes depending upon the amount of material used. The probable error was less than 5 per cent of the mean, except at the last point with pH 1.95 where it was 6.5 per cent of the mean.
Fig. 2. Respiration of *Penicillium chrysogenum*. The curves show rate plotted against pH, and represent the same data as in Fig. 1. Curve A represents the respiration at the end of 8 minutes in the solution; Curve B at the end of 20 minutes; Curve C at the end of 30 minutes; and Curve D at the end of 60 minutes.
These experiments showed that in acid solutions the rate of respiration was increased at certain concentrations. The next step was to determine the effect of alkaline solutions.

The first concentration to be tried was pH 8. As it was found that this concentration had no effect on respiration the normal rate in the succeeding experiments was measured between pH 7.52 and 7.25 as the color change in phenolsulfonephthalein is much easier to determine at this range.

The next concentration chosen was pH 9. As phenolsulfonephthalein is not sensitive in this range phenolphthalein was used instead; this indicator is not toxic to the fungus. The concentrations were first standardized and it was found that the same amount of CO₂ caused a change from pH 7.52 to 7.25 and from 8.92 to 8.68. The time required for normal respiration to change the solution from pH 7.52 to 7.25 was determined; then the fungus was put in a solution slightly higher than pH 8.92 and the time required to change from pH 8.92 to 8.68 noted. It was found to be much longer than the normal, thus showing that respiration was decreased. Not only the first reading but all the succeeding ones showed a decrease, and at no time was there an increase.

When the pH value was less than 7 and there was a decrease to considerably below normal, no recovery back to normal (or nearly normal) followed, after the material had been replaced in ordinary nutrient solution at pH 4 or in sugar solution at pH 7. After the respiration had been depressed to 60 per cent of normal by pH 8.80 and kept there for an hour, there was nearly complete recovery when put back in sugar solution at pH 7. It is therefore evident that any considerable decrease produced by acid solutions is irreversible, while a similar decrease produced by alkaline solutions is reversible.

Some experiments have also been made by measuring the consumption of oxygen by the fungus. Winkler's method, as modified by Osterhout and Haas,¹⁷ was employed.

It was found that in a solution of pH 9 the fungus uses less than one-half as much oxygen as in a neutral solution, while in a solution of pH 2 the consumption of oxygen is nearly four times as great as

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in a neutral solution. These experiments confirm the data obtained by measuring the production of CO₂, and show that in an acid medium the increase in oxygen consumption is greater than the increase in the production of CO₂, while in alkaline solution the two are about the same.

In all cases control experiments were made with solutions containing no fungus but having the same pH values as the solutions containing the fungus.

The results indicate that the increased production of CO₂ in acid solution is due to respiration and not to the action of the reagent in setting free CO₂ previously stored in the tissue (in the form of carbonates).

The work of certain investigators might lead to the expectation that, in general, moderate concentrations of acid would decrease respiration, while moderate concentrations of alkali would increase it. It is evident that the organism here studied behaves in the opposite manner. This raises some interesting questions regarding the reactions in the organism which result in the production of CO₂; further investigation will be necessary to clear up these questions. It is possible that the results obtained from the study of Penicillium are connected with the fact that it grows best on an acid medium. In the experiments of the writer spores grew best on media with a pH value of from 4 to 6.

SUMMARY.

1. Variations in pH value between 4 and 8 produce practically no effect on the normal rate of respiration (the rate at neutrality is called normal).

2. Increasing the pH value to 8.80 causes respiration to fall to 60 per cent of the normal, after which it remains stationary for the duration of the experiment.

3. Decreasing the pH value to 2.65 causes a gradual rise and a gradual return to normal; at pH 1.10 to 1.95 the preliminary rise amounts to 20 per cent and is followed by a fall to below the normal.

4. The decrease in respiration brought about by solutions of a pH value of 1.95 or less are irreversible, while a similar decrease
which occurs at pH 8.80 is reversible, the rate coming back to practically normal after the material is replaced in a neutral solution.

5. Determinations by means of Winkler's method showed an increase in the consumption of oxygen in acid solutions and a decrease in alkaline solutions.