COMPARATIVE STUDIES ON RESPIRATION.

VIII. The Respiration of Bacillus Subtilis in Relation to Antagonism.

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Although the antagonistic effects of salts on certain bacteria have been studied, no attempt has been made to ascertain whether any relation exists between these effects and the respiration. The investigations described in this paper were undertaken with a view to obtaining some light on this question.

The first attempt to study antagonism in relation to bacteria was made by Lipman.[1] He used the production of NH_3 by *Bacillus subtilis* as an index of metabolism. He allowed cultures of *Bacillus subtilis* to grow over night in various salts and combinations of salts in the proportions found in sea water, and measured the production of NH_3. There was a considerable decrease in the production of NH_3 when the salts were used singly, but this did not occur when combinations of salts were made in the proportions found in sea water.

Winslow and Falk[2] have observed antagonistic effects in experiments on *Bacillus coli*. These investigators found that cultures suspended in solutions of NaCl or $CaCl_2$ were decreased in number; that higher concentrations produced sterilization of the culture; and that a combination of NaCl and $CaCl_2$ in the molecular proportions of 5 : 1 was favorable to the growth of the organism.

[1] Lipman, C. B., *Bot. Gaz.*, 1909, xlviii, 105; 1911, xlix, 41.

[2] Winslow, C.-E. A., and Falk, I. S., *Proc. Soc. Exp. Biol. and Med.*, 1918, xv, 67.

Shearer[3] has also demonstrated similar effects of salts upon the viability of meningococcus and *Bacillus coli*. He found that a combination of NaCl and $CaCl_2$ was favorable to growth, while each salt used separately produced decrease in growth.

It is evident, therefore, that antagonistic effects are to be expected in studying bacteria, and it seemed desirable to investigate these effects in relation to respiration. For this purpose the apparatus described by Osterhout[4] was employed.

The organism selected was *Bacillus subtilis*, the same strain as that used in an investigation previously reported.[5] It had originally been isolated from water and grown daily upon agar-agar. In every case an 18 hour culture, inoculated upon agar-agar with a few cc. of 0.75 per cent dextrose solution, was used. These inoculations were incubated at 37°C. and the resulting heavy growth of bacteria was washed off with 0.75 per cent dextrose solution and centrifugated to get rid of any foreign substances that may have surrounded the bacteria. The bacteria were then suspended in 0.75 per cent dextrose solution and were ready for experimentation. It is very important to use cultures not older than 24 hours, as the respiration of the older cultures is considerably diminished.

In making up the solution of dextrose and the salts, distilled water was employed. The salts used were NaCl, $CaCl_2$, and KCl in molecular concentrations from 0.05 M to 1.0 M. Experiments on $MgCl_2$, which belong to this series, are in process of completion.

The temperature varied in the course of the experiments from 18–20°C.

The indicator used was 5 drops of 0.01 per cent phenolsulfonephthalein in 10 cc. of water. Tap water was used, as distilled water has a pH value not exceeding 7.1 and this was not alkaline enough for measuring with this indicator. Buffer solutions were made from boric acid and borax, having pH values of 7.78 and 7.60 respectively. These buffers were used as the standard for comparison in determining the color change produced in the indicator as the CO_2 was driven over.

[3] Shearer, C., *Proc. Roy. Soc. London, Series B*, 1917, lxxxix, 440; *Proc. Camb. Phil. Soc.*, 1919, xix, 263.

[4] Osterhout, W. J. V., *J. Gen. Physiol.*, 1918–19, i, 17, 171.

[5] Brooks, M. M., *J. Gen. Physiol.*, 1918–19, i, 193.

For experimentation, 2 cc. of the emulsion of bacteria were placed in the apparatus, and the air was caused to circulate. The CO_2 produced by the bacteria was thereby carried over into the indicator, and the reciprocal of the time required to change the color from pH 7.78 to 7.60 was taken as the rate of respiration or production of CO_2. When the indicator had reached the end-point required, the stop-cock was closed, thus allowing the CO_2 to be washed out of the system again by passing the air through the NaOH, and thereby returning the indicator to its original color. In this way, a series of readings could be taken. In general these were remarkably constant. It was found that the rate of respiration, under normal conditions (*i.e.* of bacteria placed in 0.75 per cent dextrose solution), was practically constant for about 6 hours. The experiments, however, lasted only 70 minutes. This included the time necessary for the establishment of the normal rate, usually 10 minutes, and the time for determining the effect of the salt.

When the normal rate had been determined, the salt was added and the change in the rate was observed. The first reading was discarded owing to the possibility of experimental error, as the result of CO_2 dissolved in the salt solution. In adding the salt solution, the system was opened because it was found that no appreciable error was introduced by exposing the system for a moment to contact with the air.

When the bacteria had been in contact with the salt for an hour, the respiration seemed to have reached an equilibrium, as the rate then decreased very slowly during the next few hours. This decrease was faster in the higher concentrations of the salt; the lower concentrations remained constant for hours at a time. In adding the salt, 2 cc. (of double the strength desired for experimentation) were added to 2 cc. of the bacteria in 0.75 per cent dextrose solution, so that the volume remained constant throughout all the experiments.

It is generally known that a reduced pressure of oxygen has little effect for a considerable time upon the rate of oxidation, so that the slight change in the oxygen content of the system during the experiment does not introduce an experimental error.

Fig. 1 shows the manner in which the rate of CO_2 production changes under the influence of NaCl in the concentrations of 0.15,

0.1, 0.5, 0.8, and 1.0 M. During the first 10 minutes the bacteria are under normal conditions and the curve (broken line) is horizontal. After this (at the point marked 0 on the abscissa) the salt is added.

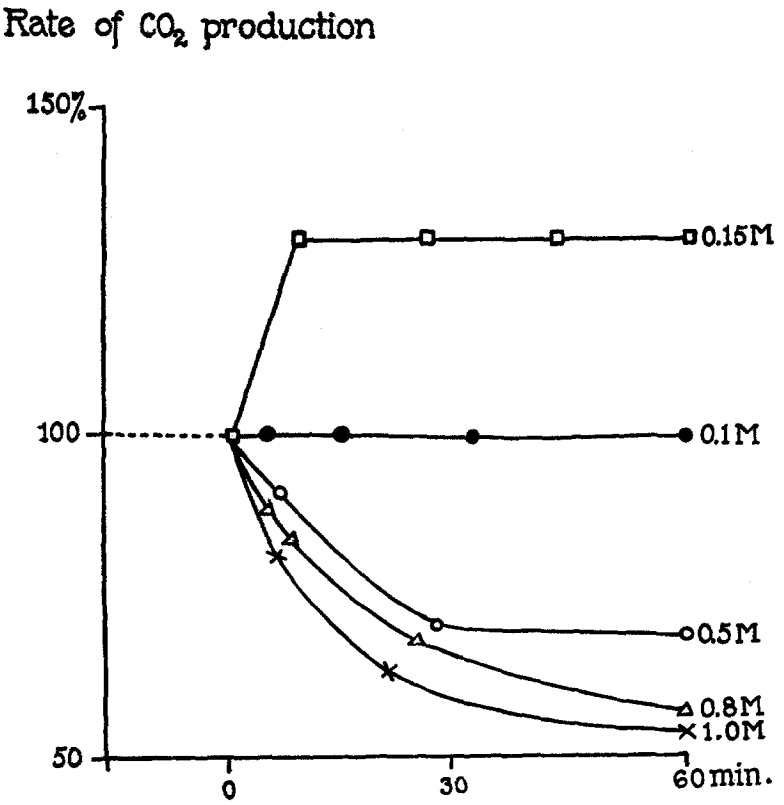

FIG. 1. Curves showing the rate of respiration of *Bacillus subtilis* (expressed as per cent of the normal) in 0.15, 0.1, 0.5, 0.8, and 1.0 M NaCl. The zero point on the abscissa denotes the beginning of exposure to the salt solution; previous to this the bacteria were in 0.75 per cent solution of dextrose in distilled water. The normal rate (which is taken as 100 per cent) represents a change in pH value from 7.78 to 7.60 in a number of seconds depending upon the amount of bacterial suspension used, usually 30 seconds. Each curve represents a single typical experiment.

For example, the addition of sufficient NaCl to make the concentration 0.15 M produces a rise in the rate, which remains constant during the period of experimentation. When the concentration of NaCl

is 0.1 M the rate is normal, while in higher concentrations there is a decrease in rate. These curves are selected from a number of similar typical curves, and each represents one experiment.

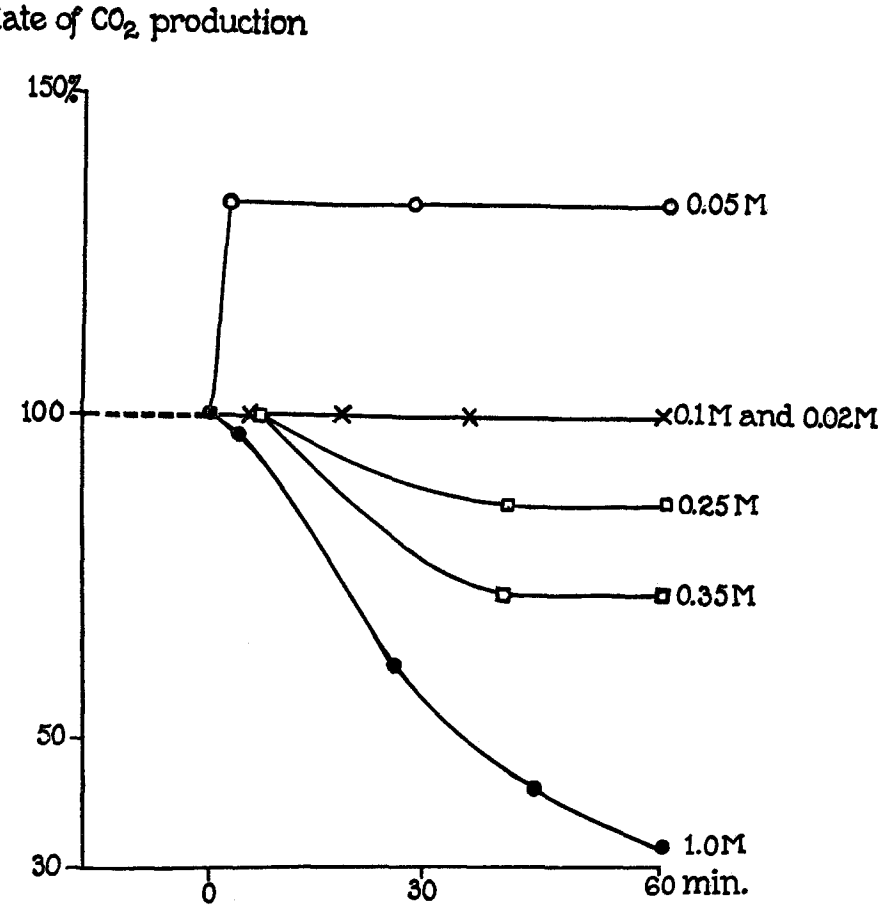

FIG. 2. Curves showing the rate of respiration of *Bacillus subtilis* (expressed as per cent of the normal) in 0.05, 0.1, 0.25, 0.35, and 1.0 M $CaCl_2$. The zero point on the abscissa denotes the beginning of exposure to the salt solution; previous to this the bacteria were in 0.75 per cent solution of dextrose in distilled water. The normal rate (which is taken as 100 per cent) represents a change in pH value from 7.78 to 7.60 in a number of seconds depending upon the amount of bacterial suspension used, usually 30 seconds. Each curve represents a single typical experiment.

Fig. 2 shows the manner in which the rate of CO_2 production changes under the influence of $CaCl_2$ in the concentrations of 0.02, 0.05, 0.1, 0.25, 0.35, and 1.0 M. In 0.01, 0.02, and 0.1 M the rate is normal; in 0.05 M there is an increase in rate; in higher concentrations there is a decrease in rate. These curves are selected from a number of similar typical curves, and each represents one experiment.

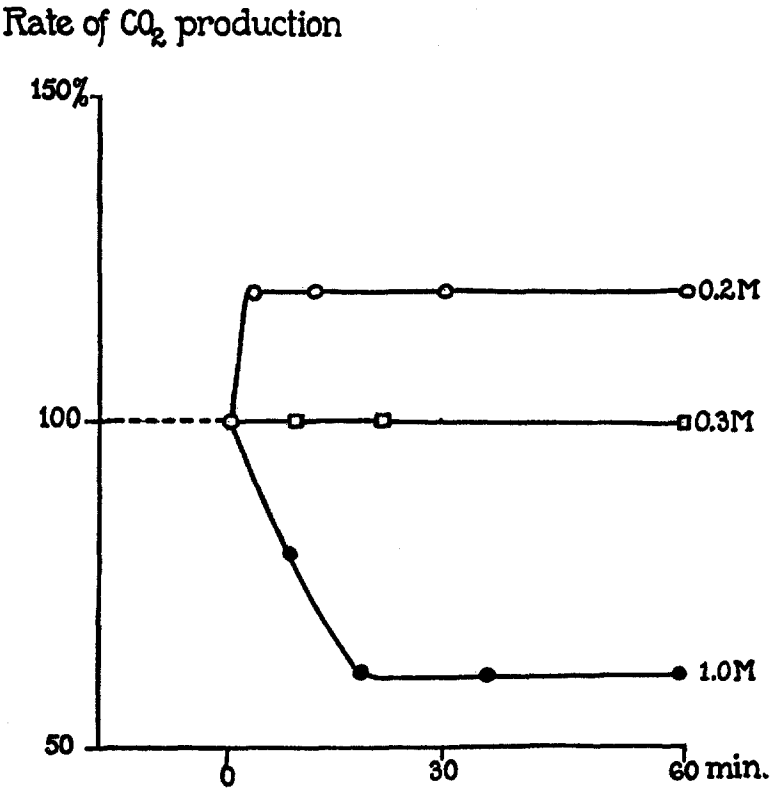

FIG. 3. Curves showing the rate of respiration of *Bacillus subtilis* (expressed as per cent of the normal) in 0.2 M, 0.3 M, and 1.0 M KCl. The zero point on the abscissa denotes the beginning of exposure to the salt solution; previous to this the bacteria were in 0.75 per cent solution of dextrose in distilled water. The normal rate (which is taken as 100 per cent) represents a change in pH value from 7.78 to 7.60 in a number of seconds depending upon the amount of bacterial suspension, usually 30 seconds. Each curve represents a single typical experiment.

Fig. 3 shows the manner in which the rate of CO_2 production changes under the influence of KCl in concentrations of 0.2, 0.3, and 1.0 M. When the concentration is 0.2 M, there is an increase in the rate which remains constant for some time; in lower concentrations (0.15 and 0.1 M) the rate is normal, while in higher concentrations there is a decrease in rate. These curves are selected from a number of similar typical curves, and each represents one experiment.

Fig. 4 shows the effects of various concentrations of NaCl, KCl, and $CaCl_2$ upon the rate of respiration expressed as per cent of the normal rate. The rate indicated is that produced after the bacteria had been in contact with the salt for 1 hour. The figure shows that NaCl produces an increase in the rate of respiration at a concentration of 0.15 M. In 0.5, 0.8, and 1.0 M there is a decrease. KCl produces an increase in the rate at a concentration of 0.2 M, and in concentrations higher than 0.3 M it causes a decrease. $CaCl_2$ causes an increase in the rate at a concentration of 0.05 M and in concentrations higher than 0.1 M it causes a decrease in respiration. $CaCl_2$ is the most toxic of the salts used, while KCl is the least toxic; this agrees with the results of Lipman.[1] It is of interest to note that there is evidently a correlation between the production of NH_3 of the organism (as found by Lipman) and the rate of production of CO_2 as shown here.

Fig. 5 shows the antagonism of salts. Thus, Curve A shows that when five parts of NaCl and one part of $CaCl_2$ (in the same molecular concentrations) were added to the bacteria, the rate of respiration remained normal, or as if no salt had been added. This was true only when the proportions of 5:1 were used. When other proportions were used the respiration decreased accordingly, and gave only a fraction of the normal rate. This agrees with the results of Winslow and Falk[2] on the growth of *Bacillus coli*, but not with those of Lipman,[1] on the production of NH_3 by *Bacillus subtilis*, who found no antagonism between NaCl and $CaCl_2$.

Curve B illustrates the effect of combinations of KCl and NaCl upon the rate of respiration. There are two maxima in this curve; one at 4 KCl to 6 NaCl, and the other at 6 KCl to 4 NaCl. The former is the more nearly normal, although there is no combination

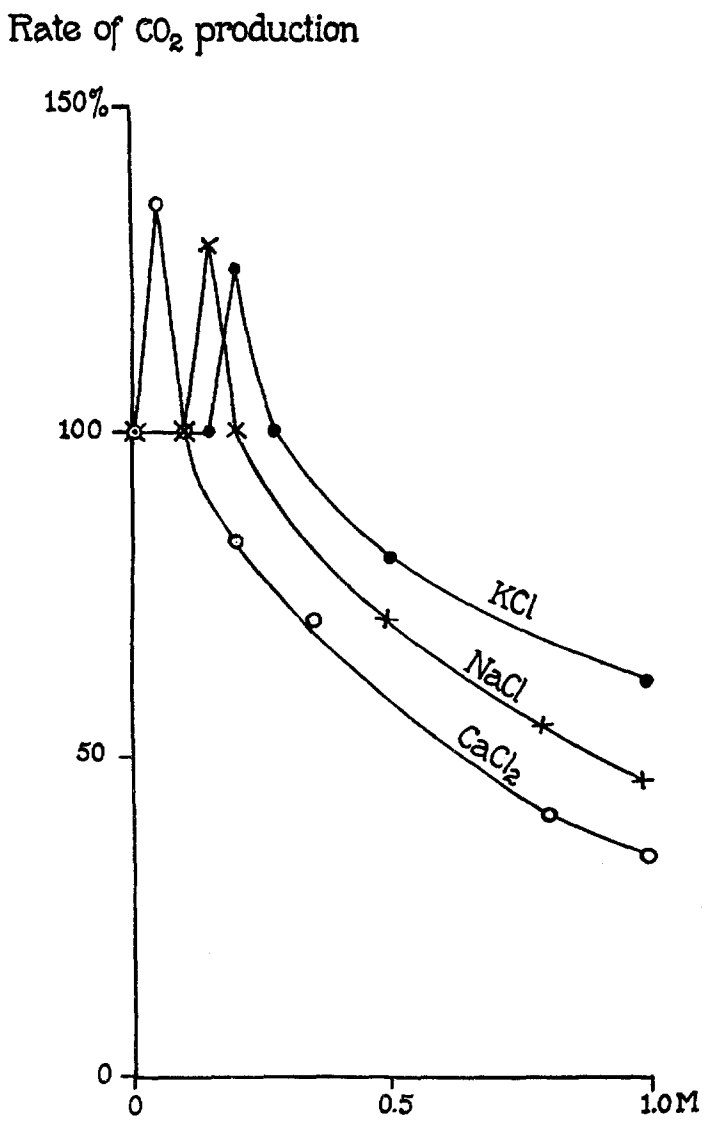

FIG. 4. Curves showing the rate of respiration of *Bacillus subtilis* (expressed as per cent of the normal) as effected by salts. The normal rate (which is taken as 100 per cent) represents a change in pH value from 7.78 to 7.60 in a number of seconds depending upon the amount of bacterial suspension used, usually 30 seconds. Average of three experiments; probable error less than 3 per cent of the mean.

of these two salts (in the concentrations used) that produces normal respiration. It is of interest to note that Osterhout[6] and Lipman[1] also obtained two maxima with these salts in experiments upon wheat

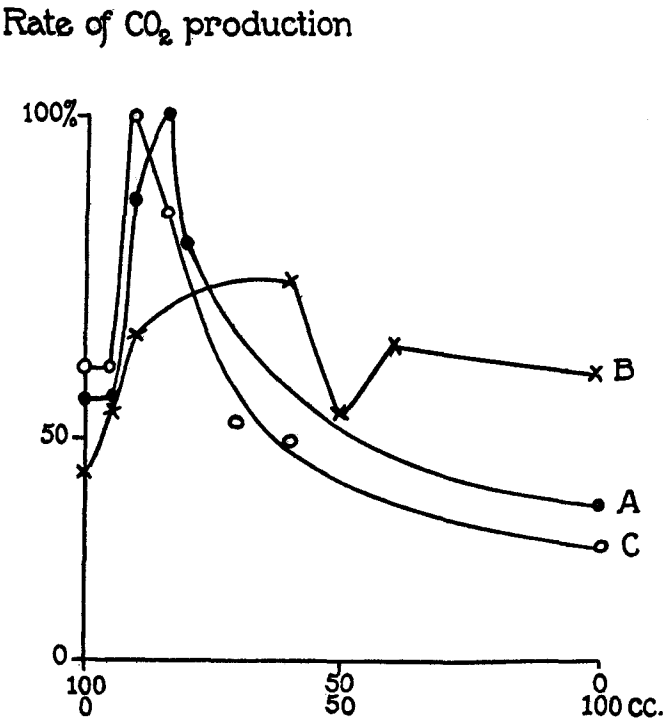

FIG. 5. Curves showing antagonism in the effect of salts on the respiration of *Bacillus subtilis*. Curve A, antagonism between NaCl, 0.8 M (left), and $CaCl_2$, 0.8 M (right); Curve B, antagonism between NaCl, 1 M (left), and KCl 1 M (right); Curve C, antagonism between KCl, 1 M (left), and $CaCl_2$, 1 M (right). The ordinates represent rate of respiration (expressed as per cent of the normal); the abscissæ represent molecular proportions of the salts used. Thus, in Curve A, the ordinate at the extreme left represents the rate in NaCl 0.8 M, while the ordinate at the extreme right represents the rate in $CaCl_2$ 0.8 M. The ordinate in the middle represents the rate in 50 parts NaCl 0.8 M + 50 parts $CaCl_2$ 0.8 M. The normal rate (which is taken as 100 per cent) represents a change in pH from 7.78 to 7.60 in about 30 seconds, varying according to the number of bacteria used. Curve A, average of two experiments; Curve B, average of five experiments; Curve C, average of three experiments. Probable error less than 3 per cent of the mean.

[6] Osterhout, W. J. V., *Bot. Gaz.*, 1909, xlviii, 98.

and upon the production of NH_3 by *Bacillus subtilis* respectively. The fact that both salts are monovalent may be a factor in explaining their peculiar behavior.

Curve C of Fig. 5 shows antagonism between KCl and $CaCl_2$. This was also observed by Lipman[1] in the production of NH_3. The maximum effect is found at 9 KCl to 1 $CaCl_2$, where the rate of respiration is 100 per cent. In comparing this curve with Curve A (NaCl and $CaCl_2$) one can readily observe that more KCl is required to antagonize $CaCl_2$ than would be required of NaCl. It is interesting to observe that KCl is the least toxic of the three salts, and that it is the least effective in influencing respiration. This agrees with the results of Lipman[1] on the production of NH_3 by *Bacillus subtilis*.

There are no similar investigations on the respiration of plants with which a comparison might be made. Some interesting studies have been made on sea urchin eggs by Warburg,[7] by Loeb and Wasteneys,[8] and by Meyerhof,[9] an account of which is given in a recent summary by Osterhout.[10] The results obtained with bacteria agree with those of Loeb and Wasteneys in that there is no rise in rate in NaCl, except in 0.15 M concentration in which the rise is only 30 per cent which is insignificant compared with that obtained by Warburg[7] and Meyerhof[9] (200 to 500 per cent). On the whole the results are more nearly in agreement with those of Loeb and Wasteneys.[8]

In order to find out what effect was produced on the bacteria while they were being acted upon by the salts, a few recovery experiments were tried. After the bacteria had remained in the salt solution for an hour, they were centrifugated and thoroughly washed in dextrose solution and centrifugated again. The supernatant fluid was then drained off, 2 cc. of dextrose solution were added, and their respiration was measured. It was found that within a period of less than $\frac{1}{2}$ hour the rate became normal.

A control experiment was made by substituting the same salt in the same molecular concentration as was removed from the bacteria

[7] Warburg, O., *Z. physiol. Chem.*, 1910, lxvi, 305; *Biochem. Z.*, 1910, xxix, 414.
[8] Loeb, J., and Wasteneys, H., *Biochem. Z.*, 1910, xxviii, 340; 1911, xxxi, 168.
[9] Meyerhof, O., *Biochem. Z.*, 1911, xxxiii, 291.
[10] Osterhout, W. J. V., *J. Gen. Physiol.*, 1919–20, ii, 1.

after centrifugating them to see whether or not the mechanical manipulation was responsible for the normal rate. The rate, however, showed no recovery under these conditions.

In order to find out whether the pH value of the liquid containing the bacteria changed when the salts were added, thereby influencing the rate of respiration, an indicator was added to this liquid in the apparatus and the pH value was observed to remain so nearly constant that the change in the rate of respiration could not be attributed to changes in alkalinity of the medium in which the bacteria were placed.

SUMMARY.

1. In relatively low concentrations of NaCl, KCl, and $CaCl_2$ the rate of respiration of *Bacillus subtilis* remains fairly constant for a period of several hours, while in the higher concentrations, there is a gradual decrease in the rate.

2. NaCl and KCl increase the rate of respiration of *Bacillus subtilis* somewhat at concentrations of 0.15 M and 0.2 M respectively; in sufficiently high concentrations they decrease the rate. $CaCl_2$ increases the rate of respiration of *Bacillus subtilis* at a concentration of 0.05 M and decreases the rate at somewhat higher concentrations.

3. The effects of salts upon respiration show a well marked antagonism between NaCl and $CaCl_2$, and between KCl and $CaCl_2$. The antagonism between NaCl and KCl is slight and the antagonism curve shows two maxima.