THE RELATION OF THE REACTION AND OF SALT CONTENT OF THE MEDIUM ON NITRIFYING BACTERIA.*

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(Received for publication, May 30, 1922.)

The literature of soil bacteriology is replete with assertions to the effect that soil bacteria of value for the maintenance of an appropriate soil condition need an alkaline medium for their proper development. This has been repeatedly emphasized, particularly in connection with the nitrifying and the nitrogen-fixing flora of the soil. Actual evidence substantiating such assertions has never been adduced from experiments, or at least, it has never been presented. This situation is, in part, explained by the vagueness of our old conceptions with regard to the real nature of alkalinity and acidity as applied to biological media. It is natural, therefore, that studies such as those needed to give the subject a proper status should have lagged behind the rapid development of the methods for determining hydrogen and hydroxyl ion concentrations in the last 8 to 10 years. Even such a recent investigation as that of Stephenson (1) on the relations of nitrification to acidity in soils takes no account of the

* Since this paper was written, there has come to our notice the study of Meyerhof (Meyerhof, O., Arch. ges. Physiol., 1916, clxiv, 353), on the respiration of nitrate-forming bacteria. Meyerhof found that the optimum pH for the nitrate former was between 8.3 and 9.3. This is considerably below the figure obtained by us as described in this paper. Moreover, Meyerhof's establishment of definite lethal points at pH values of 5.6 and 10.3 for hydrogen and hydroxyl ion, respectively, seem to us to be based on insufficient evidence, inasmuch as we show in this paper that the strain of the nitrifying bacteria as determined by its source is an important factor of the lethal point of reaction. Finally, the actual resistance to hydroxyl ion as judged by ability to live (even though inactive), is found by us to be much greater than that indicated by Meyerhof's results. In general, however, it is gratifying to find that the two studies confirm each other on the point that nitrifying bacteria are highly resistant to hydroxyl ion and are, in general, less resistant to hydrogen ion.
actual hydrogen ion concentrations involved, but only of acidity expressed in the terms of more than a decade ago.

In view of the foregoing, we deemed it necessary to determine, if possible, just what the relations of nitrifying bacteria to the reaction of their medium may be. In addition, another subject connected with the nitrifying bacteria seemed to call for our attention, and that was the relations of nitrifying bacteria to high salt concentrations. One of us (2) had already investigated the latter subject by using soils as media. That work, however, was done some years ago without due appreciation of the uncontrolled variables involved in the use of soils as media. For that reason, it seemed highly desirable to study the subject of salt concentrations again and this time in culture solutions of simple and known concentration.

Experiments on Reaction of the Medium.

The culture solution employed in these experiments was constituted as follows:

\[
\begin{align*}
1,000 \text{ cc.} & \text{ distilled water.} \\
1 \text{ gm.} & \text{ K}_2\text{HPO}_4 \\
1 \text{ "} & \text{ NaCl} \\
0.5 \text{ "} & \text{ MgSO}_4 \\
1 \text{ drop of 10 per cent solution of FeCl}_3 \\
\end{align*}
\]

To this solution was added, as needed, 1 gm. of (NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4} per liter for nitrite formation cultures and 1 gm. of NaNO\textsubscript{3} per liter for nitrate formation cultures. Varying amounts of NaOH and H\textsubscript{3}PO\textsubscript{4} were added to the different series of cultures to provide a wide range of hydroxyl and hydrogen ion concentrations, respectively. The actual concentrations of these ions in every culture were determined either by the hydrogen electrode or by the colorimetric method, of Clark and Lubs, and in many instances by both methods, used so as to check each other. Determinations of the hydrogen and hydroxyl ion concentrations were made at frequent intervals during the incubation period. When solutions of low concentration of hydrogen ion were desired, K\textsubscript{2}HPO\textsubscript{4} was employed. In order to insure a good buffer effect, the 14.25 normal NaOH which we used, was made up with a K\textsubscript{2}HPO\textsubscript{4} solution, instead of with distilled water.
Difficulties encountered in attempts to obtain pure cultures of the nitrifying bacteria led us to adopt the use of a crude, but invigorated culture in each case, which was produced by numerous successive transfers into fresh media from a strong soil culture in the solutions above described. The media were distributed in 25 cc. portions in 250 cc. Erlenmeyer flasks and plugged and sterilized as is customary. The cultures were run in quadruplicate and the series in duplicate. Incubation was carried out at a temperature of 28°C. Only qualitative tests were made for nitrites and nitrates, the Trommsdorff reagent and the diphenylamine tests being employed. At the end of the first 5 days of the incubation period and on every succeeding day, every culture was tested. Sterile distilled water was added from time to time to maintain constant concentrations. In the three different series of our experiments, different soils were employed for obtaining the necessary bacterial cultures. These soils were a garden soil, a blow-sand soil, and a peat soil. We assumed that the enrichment cultures which we obtained by several months of culturing by the Gibbs' method (3) could be justifiably used because any contaminating organisms present could not be expected to exert any influence on the nitrifying bacteria in the peculiar solutions used. Since the results obtained were similar in the different soils, we present here only the data for the garden soil organisms. In the case of the peat soil, we give the data on nitrite formation separately, since they differed from those of the garden soil. In Table I will be found the results obtained with the nitrate-forming bacteria operating in mixed cultures, but in solutions which presumably prevent other bacteria from working. In Table II, we present the results obtained from the garden and the peat soil cultures of the nitrite- forming organisms. Where bacterial action was positive, indication thereof is given in the tables by a plus sign; where it was negative by a minus sign.

The data in the tables furnish most interesting and unexpected results. In comparing the hydrogen and hydroxyl ion concentrations, expressed in our tables by pH values, for the freshly inoculated cultures with those first showing nitrification, we note the very striking fact that both nitrite- and nitrate-forming organisms from the garden soil can withstand extremely high concentrations of hydroxyl ion,
in one case pH 13.0 and in the other 13.1. This exceeds the resistance of any living organism, of which we have knowledge, to the effects of alkalinity. In the second place, the comparison in question confirms the findings of several investigators to the effect that living cells modify very rapidly the reaction of the media into which they are introduced. This seems to be a universal phenomenon among living cells. While, therefore, both the nitrite- and nitrate-forming bacteria originating from the garden soil could live and function in solutions whose pH was 13.0 and 13.1, respectively, at the time when they began to yield their characteristic products they had reduced the pH value of their media to 10.3 in one case, and to 10.0 in the other. Even that corresponds to a hydroxyl ion concentration, however, which is in excess of that tolerated by any other organisms.

TABLE I.

<table>
<thead>
<tr>
<th>pH values.</th>
<th>At beginning</th>
<th>At time of nitrification</th>
<th>Nitrification</th>
</tr>
</thead>
<tbody>
<tr>
<td>14</td>
<td>14</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>13.8</td>
<td>13.8</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>13.4</td>
<td>13.4</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>13.1</td>
<td>10.0</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>12.8</td>
<td>9.9</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>12.7</td>
<td>10.3</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>12.5</td>
<td>9.6</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>12.3</td>
<td>9.8</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>11.6</td>
<td>9.6</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>11.1</td>
<td>9.4</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>10.5</td>
<td>9.0</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>9.5</td>
<td>8.6</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>8.8</td>
<td>8.6</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>8.5</td>
<td>8.4</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>7.2</td>
<td>6.6</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>7.1</td>
<td>6.6</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>7.0</td>
<td>6.6</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>6.6</td>
<td>6.5</td>
<td>+</td>
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</tr>
<tr>
<td>6.2</td>
<td>6.2</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>5.6</td>
<td>5.6</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>5.4</td>
<td>5.3</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>5.3</td>
<td>5.3</td>
<td>-</td>
<td></td>
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</tbody>
</table>
of which we have knowledge. It is significant, further, that at the lower concentrations of NaOH employed, there was relatively little

TABLE II.

<table>
<thead>
<tr>
<th>pH values</th>
<th>Nitrification</th>
</tr>
</thead>
<tbody>
<tr>
<td>At beginning</td>
<td>At time of nitrification</td>
</tr>
<tr>
<td>Series 2. <em>Nitrosomonas</em> garden soil.</td>
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<tr>
<td>13.4</td>
<td>13.4</td>
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<tr>
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<td>10.0</td>
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<td>10.0</td>
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<td>5.4</td>
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<tr>
<td>4.7</td>
<td>4.7</td>
</tr>
<tr>
<td>4.3</td>
<td>4.3</td>
</tr>
<tr>
<td>4.2</td>
<td>4.2</td>
</tr>
</tbody>
</table>


| 13.0 | 9.3 | + |
| 10.0 | 7.8 | + |
| 9.5  | 7.1 | + |
| 8.0  | 6.8 | + |
| 7.2  | 6.8 | + |
| 6.4  | 6.3 | + |
| 6.2  | 6.2 | + |
| 6.0  | 6.0 | + |
| 5.4  | 5.2 | + |
| 4.7  | 4.6 | + |
| 4.3  | 4.2 | + |
| 4.2  | 4.1 | + |

change in the hydroxyl ion concentration from the initial value to that characterizing the solution when nitrite formation was observed.
That was not so true of nitrate formation. The extreme resistance of these organisms as noted above was further tested after they had shown their characteristic activities in the cultures of pH values of 13.0, and had reduced these cultures to pH 10.0; more alkali was then added, bringing the hydroxyl ion concentration up to the original value, but nitrification was thereby stopped. That the process did not later repeat itself as before was doubtless due to the high osmotic concentration of the solution which resulted from the addition of these large quantities of alkali. In the case of the peat soil organisms, we find much less resistance to hydroxyl ion concentration than in the garden soil and no nitrate formation is found at pH values above 9.5. Even those organisms, however, produced nitrites from ammonia at a pH of 9.3, and while this shows considerable variability as compared with the nitrifying bacteria from the garden soil, it strengthens the evidence on the most striking point presented in our data, to the effect that the resistance of nitrifying bacteria to high hydroxyl ion concentrations is exceptional in the living world.

On the acid side of the neutral point, our observations furnish the following data. The organisms from the garden soil stop the production of both nitrites and nitrates at pH values below 5.4. Again we find a difference between the organisms from the garden and the blow-sand soil, which always behaved exactly alike, and those from the peat soil. Not only did the latter go on producing nitrites from ammonium salts in solutions with a pH below 5.4, but continued doing so at a pH of 4.1 which was the most acid medium employed by us. Doubtless their resistance to acidity can be shown to be much greater, but new experiments on that point would have to be arranged, providing for the use of an acid which dissociates to a much greater degree than the \( \text{H}_3\text{PO}_4 \) which we used, so that small increases in concentration of acid would suffice to allow the requisite range of hydrogen ion concentration. Since the reason for our using \( \text{H}_3\text{PO}_4 \) must be obvious to the reader, no further discussion of those points is necessary here. It may not be out of place, however, to mention a difficulty in a problem like ours in connection with the use of high concentrations of acid; that is, that nitrous acid is oxidized under those conditions to nitric acid without the intervention of the bacteria. That necessarily limits us to certain ranges of hydrogen ion concentration.
Recurring now to the results obtained with the organisms of the peat soil, it becomes evident that they cannot withstand as much hydroxyl ion but can resist much more hydrogen ion than the nitrifying bacteria from the other soils. Since the peat soil is naturally acid in reaction (pH about 4.6), it is of interest to ascertain whether in this case a marked impression is made upon an organism by its environment even though such impression does not produce a change in the character of its fundamental functions. The nitrifying bacteria will probably be traced back some day to a common source. If that should be the case, our data will furnish one step in the line of reasoning which may prove the marked influence of the environment on living cells.

As regards a further comparison between the nitrite and nitrate formers, it should be added that, while our data are not conclusive on that point, they would seem to indicate, in general, that the nitrate-forming bacteria are slightly more resistant to alkalinity than the nitrite-forming bacteria.

It seems of interest to consider the rate at which the nitrate-forming bacteria produce nitrates from nitrites under varying concentrations of hydrogen and hydroxyl ion, other conditions being equal throughout. Fig. 1 gives in graphic form the results of such a study. The curve indicates the amount of NaNO₂ (in milligrams) which was oxidized to nitrate at different pH values of the culture solutions. It will be seen from the curve that the qualitative results of the tables are confirmed, the maximum velocity of reaction being noted at pH 10.0. This, therefore, seems to be the optimum point for the speedy oxidation of nitrite to nitrate. The rate of increase from pH 6.1 is extremely rapid up to pH 7.1. It then becomes much slower until the maximum increase is reached at pH 10. Beyond the latter point, there is a gradual drop in the rate until an initial pH of 13.1 is reached, in which culture 237 mg. of NaNO₂ are oxidized in 32 days.

The Resistance of Nitrifying Bacteria to High Salt Concentrations.

This part of the investigation was preliminary in nature and sought to establish some definite ranges of salt concentration within
which nitrification by the organisms in question proceeds actively and without which it ceases completely. Therefore, three sodium salts were employed at a few concentrations only with the results indicated in Table III. The plus sign indicates active nitrification; the minus sign, no nitrification. The culture solutions were made up in Erlenmeyer flasks as in the experiments described above and the organisms were grown (not in pure, but in invigorated crude cultures) in thin layers of solution. The salts were added as above indicated.
The data of Table III seem to justify the conclusion that the toxicities of NaCl and Na₂CO₃ to nitrifying bacteria are of about the same order of magnitude, but that Na₂SO₄ is not nearly so toxic as either of the other salts. The problem is more complicated in the case of the Na₂CO₃ series inasmuch as the hydroxyl ion concentration was greater in the solutions containing it than in the solutions supplied with the other salts. This effect must, however, have been offset more or less completely by the marked buffer action of the nitrifying cells which is discussed in the first part of this paper. The results in Table III suggest the immediate need for more detailed experiments along this line, which we hope to carry through. It should be added, however, that since work of the kind reported in this paper is the first reported for simple culture solutions with nitrifying bacteria, and since such cultures are much simpler and involve fewer variables than soil cultures, our results must be regarded as more reliable than the earlier ones obtained by one of us and by other investigators who have dealt with the problem more recently. While the findings of the present paper agree with earlier ones regarding the relative toxicity of NaCl and Na₂SO₄ to nitrifying bacteria, the

<table>
<thead>
<tr>
<th>TABLE III.</th>
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<tbody>
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<td>Nitrification.</td>
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<table>
<thead>
<tr>
<th>NaCl series.</th>
</tr>
</thead>
<tbody>
<tr>
<td>10,000 parts per million (0.171 N)</td>
</tr>
<tr>
<td>20,000 &quot; &quot; (0.282 N)</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Na₂CO₃ series.</th>
</tr>
</thead>
<tbody>
<tr>
<td>5,000 parts per million (0.094 N)</td>
</tr>
<tr>
<td>10,000 &quot; &quot; (0.188 N)</td>
</tr>
<tr>
<td>20,000 &quot; &quot; (0.276 N)</td>
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</tbody>
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<table>
<thead>
<tr>
<th>Na₂SO₄ series.</th>
</tr>
</thead>
<tbody>
<tr>
<td>10,000 parts per million (0.140 N)</td>
</tr>
<tr>
<td>20,000 &quot; &quot; (0.280 N)</td>
</tr>
<tr>
<td>25,000 &quot; &quot; (0.350 N)</td>
</tr>
<tr>
<td>30,000 &quot; &quot; (0.420 N)</td>
</tr>
<tr>
<td>35,000 &quot; &quot; (0.990 N)</td>
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other features of the results are very different and for the reason
given above should be considered more dependable. In all cases,
it is very striking to note the marked tolerance for high salt concen-
trations manifested by the nitrifying bacteria.

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