THE EFFECT OF REACTION ON THE FIXATION OF NITROGEN BY AZOTOBACTER*

BY
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INTRODUCTORY

Azotobacter has been given considerable attention in the literature of soil bacteriology and it is thought that it plays an important part in keeping up the supply of nitrogen in many soils.

It has been recognized, almost since its first discovery, that Azotobacter is especially sensitive to the reaction of the medium in which it grows. The effect of acidity on the organism is so well established that its absence from a soil has frequently been taken as an indication that the soil needed lime. In fact, Christensen has worked out a method wherein he uses these organisms to determine the lime requirement of soils. The point has been repeatedly stressed that the acidity of soils must be neutralized if Azotobacter is to fix nitrogen effectively.

Since the influence of hydrogen-ion concentration on bacteria has been recognized, the following investigators have reported on its effect on these organisms.

Fred and Davenport\(^1\) reported that Azotobacter is very sensitive, the limits for its growth being between P\(_H\) 6.6 and P\(_H\) 8.4 to 8.8.

Gainey\(^2\) in a preliminary report states that in 90 soils studied all but 3 of the 37 in which no Azotobacter was found had a P\(_H\) value of 5.9 or less and all but 3 of those containing the organisms had P\(_H\) values of 6.0 or greater. In later papers\(^3\) he reported that Azotobacter rapidly disappears when inoculated into soils whose P\(_H\) value is below 6.0 and that, in a study of 382 soils, using the hydrogen electrode for P\(_H\) measurements, 158 samples with P\(_H\) values below 6.0 and 20 above 6.0

\* This study was undertaken at the suggestion of Dr. C. B. Lipman, in whose laboratory the work was carried out.
contained no *Azotobacter*, while 165 samples whose \( P_H \) values were above 6.0 and 39 samples below 6.0 showed *Azotobacter*. The average nitrogen fixed in 186 samples containing *Azotobacter* was 7.9 mgs., while the average fixed in 181 samples lacking the organism was 4.6 mgs.

Waksman\(^4\) in a study of cranberry soils found no *Azotobacter* in an unlimed soil with \( P_H \) values of 5.4 to 5.6, but in an adjacent limed soil with a \( P_H \) value of 6.2–6.4 found it to be present.

These investigators agree that slight acidity inhibits the growth of *Azotobacter*. None of them, however, reports on the effect of the reaction on the nitrogen fixing efficiency of the organism. Gainey reports the nitrogen fixed in the soils containing *Azotobacter* as compared with soils lacking them, but does not state the effect of the reaction in the soils where they were present.

The work here presented was undertaken to determine the effect of various hydrogen-ion concentrations on the ability of *Azotobacter chroococcum* to fix nitrogen.

**EXPERIMENTAL**

The organism used was a strain of *Azotobacter chroococcum* isolated from a California soil which in previous work had been found to be very efficient in nitrogen fixation and to produce abundant pigment.

The medium was made up as follows:

- Mannite, 15.0 gms.
- \( \text{MgSO}_4 \cdot 7\text{H}_2\text{O} \), 0.2 gms.
- \( \text{NaCl} \), 0.2 gms.
- \( \text{CaSO}_4 \cdot 2\text{H}_2\text{O} \), 0.1 gms.
- \( \text{H}_3\text{PO}_4 \), 1.5 c.c.
- Distilled water, 1000 c.c.

This solution was titrated with \( \text{N/2 KOH} \) to give definite \( P_H \) values, using the hydrogen electrode in the titration. The titration curve of the medium is shown in figure 1. The inoculations were made into 100 c.c. portions of the medium in 800 c.c. Erlenmeyer flasks. To secure uniform inoculation, 50 c.c. portions of the medium in a small Erlenmeyer flask were inoculated with *Azotobacter*, and after a characteristic membrane had formed the flask was shaken vigorously, and the suspension was used as the inoculum.
Fig. 1. Titration Curve of Medium Used

PH Values

C.C. N/2 KOH Per Liter

Values
The First Series

In this series the medium was titrated to give $P_H$ values of approximately 3, 4, 5, 6, 7, 8, 9, 10, and 11. These values changed during sterilization and incubation.

Four flasks of each reaction were inoculated and incubated three weeks at 28° C.

Three of the flasks of each reaction were left undisturbed. The others were used for $P_H$ measurements, 5 c.c. being withdrawn at intervals from each for electrometric determinations.

The $P_H$ values of the solutions in these flasks at the time of inoculation and at each of the succeeding periods are shown in figure 2.

After incubating twenty days the nitrogen in each of the undisturbed flasks was determined and the amounts fixed are given in table 1.

<table>
<thead>
<tr>
<th>Series 1</th>
<th>N Fixed Mgs.</th>
</tr>
</thead>
<tbody>
<tr>
<td>$P_H$ at time of titration</td>
<td>$P_H$ at time of inoculation</td>
</tr>
<tr>
<td>3.14</td>
<td>3.47</td>
</tr>
<tr>
<td>4.10</td>
<td>4.80</td>
</tr>
<tr>
<td>5.06</td>
<td>5.31</td>
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<tr>
<td>5.98</td>
<td>6.07</td>
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<tr>
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<td>7.08</td>
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<td>8.15</td>
<td>7.69</td>
</tr>
<tr>
<td>9.01</td>
<td>7.98</td>
</tr>
<tr>
<td>10.04</td>
<td>8.08</td>
</tr>
<tr>
<td>11.03</td>
<td>8.86</td>
</tr>
</tbody>
</table>

It will be noticed that the $P_H$ values of the media were not the same when inoculated, as at the time of titration. This may be due to incomplete reaction at the time of titration, although the titration required two days for completion. It might be due to the absorption of carbon dioxide by the more alkaline media, since these media showed the greatest changes and several days intervened between sterilization and inoculation. Probably both factors had their effect. It will be noted that the solutions with $P_H$ values of 6.0 and 7.0, whose reactions remained remarkably constant, lie in the region which is shown by
the curve in figure 1 to be most highly buffered, while those with $P_H$ values between 8.0 and 11.0, which are in a poorly buffered region, show the greatest changes. The growth of Azotobacter in the more alkaline solutions also affects the reaction. The more acid solutions, although in a poorly buffered region, show little change in reaction.

Fig. 2. Changes in $P_H$ Values. First Series

The end point for nitrogen fixation on the acid side of the neutral point evidently lies very close to $P_H$ 6.0. At that reaction only a very small amount of nitrogen was fixed and in the solutions with lower $P_H$ values no fixation occurred. The alkaline reaction inhibiting fixation was not reached in this series; in fact the largest amount of $N$ was fixed in the most alkaline medium used.
The Second Series

The second series was planned to find more accurately the lower critical $P_H$ value and an alkaline reaction inhibiting fixation. Consequently solutions were made up with $P_H$ values of approximately 5, 6, 6.2, 6.4, 6.6, 6.8, 7, 8, 9, 10, 11, and 12.

Four flasks of each $P_H$ value were again inoculated and incubated, one of each $P_H$ value being used as before for $P_H$ determinations.

Only three $P_H$ determinations were made, viz., at inoculation, ten days, and seventeen days later. It is regretted that no determination was made at the end of incubation. The reactions at the time of inoculation and later are shown in figure 3. Again those in the buffered region between $P_H$ 6.0 and 7.0 showed little change, while those above in the less buffered region were markedly changed. The change in the highly buffered solution of $P_H$ 12 is remarkable, but must most likely be due to absorption of carbon dioxide since sterilization did not materially change the reaction. It should be noted that an extra flask, uninoculated, changed exactly the same as the inoculated one.

These cultures were incubated thirty days, since the amounts of nitrogen fixed in the period of twenty days in the first series were rather small. The amounts of nitrogen fixed in this series are given in table 2.

### TABLE 2

**NITROGEN FIXED IN SOLUTIONS OF VARIOUS $P_H$ VALUES**  
**Series 2**

<table>
<thead>
<tr>
<th>$P_H$ at time of titration</th>
<th>$P_H$ at time of inoculation</th>
<th>Flask No. 1</th>
<th>Flask No. 2</th>
<th>Flask No. 3</th>
<th>Average</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.00</td>
<td>5.34</td>
<td>.0</td>
<td>.0</td>
<td>.28</td>
<td>.09</td>
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<tr>
<td>5.99</td>
<td>5.97</td>
<td>.28</td>
<td>.84</td>
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<td>6.20</td>
<td>6.29</td>
<td>5.88</td>
<td>4.76</td>
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<tr>
<td>6.41</td>
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<td>6.97</td>
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</tr>
<tr>
<td>8.00</td>
<td>7.93</td>
<td>5.46</td>
<td>5.18</td>
<td>4.48</td>
<td>5.04</td>
</tr>
<tr>
<td>8.99</td>
<td>8.18</td>
<td>5.46</td>
<td>4.90</td>
<td>5.34</td>
<td>5.23</td>
</tr>
<tr>
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<td>8.52</td>
<td>5.60</td>
<td>5.88</td>
<td>.42*</td>
<td>3.97</td>
</tr>
<tr>
<td>11.02</td>
<td>9.26</td>
<td>.14</td>
<td>8.26*</td>
<td>.0</td>
<td>.07</td>
</tr>
<tr>
<td>12.05</td>
<td>11.87</td>
<td>.0</td>
<td>.0</td>
<td>.14</td>
<td>.05</td>
</tr>
</tbody>
</table>

*Not included in averages since very evidently some factor had affected the results.

The amounts of nitrogen fixed in this series were somewhat larger than those in the first series and show a somewhat different effect of reaction. In this series the neutral solution shows the greatest fixation.
It is evident again that $P_H$ 6.0 is near the critical limit for fixation. The very great increase in N fixed in the solution about .2 $P_H$ higher is remarkable. The lower limit for fixation is apparently very definite. The upper limit for nitrogen fixation was reached in the solution whose $P_H$ value at inoculation was 9.26.

Fig. 3. Changes in $P_H$ Values. Second Series
GENERAL DISCUSSION

These experiments were planned to determine the effects of hydrogen-ion concentration on nitrogen fixation by *Azotobacter*. The results obtained, however, can only be used to show that nitrogen fixation is not seriously affected until the critical limits of reaction are closely approached, when an abrupt decrease occurs in the amount of the nitrogen fixed. The variations in amounts of nitrogen fixed in solutions between these limits are not sufficient, considering the number of cultures used and the variations between cultures of the same reaction, to be of definite significance. The averages given in the tables are simply for convenience, and it is realized that a large number of flasks of each reaction would be necessary in order to secure conclusive evidence of the effects of various reactions.

The results show that there is an abrupt decrease in the amount of nitrogen fixed between $pH$ 6.2 and $pH$ 6.0, in other words, that the limiting hydrogen-ion concentration for good nitrogen fixation is a definite value between those two points. This corroborates the results of the previous investigators who found *Azotobacter* in soils whose $pH$ was 6.0 or above and none in soils with lower $pH$ values. It shows that we can expect no nitrogen fixation by *Azotobacter* in many of our soils, since it has repeatedly been shown that $pH$ values below 6.0 are frequently encountered. These organisms apparently react much more sensitively than do most of our other soil organisms, since ammonification, nitrification, and other forms of bacterial activity are active in soils whose acidity is higher than $pH$ 6.

The alkaline limit for nitrogen fixation is apparently near $pH$ 9.0, since in the first series the solution whose $pH$ value was 8.86 at inoculation showed good fixation, while in the second series the solution inoculated at $pH$ 9.26 showed no nitrogen fixed. It is doubtful whether many soils ever attain such a reaction. Sharp and Hoagland report two soils whose alkalinity is greater, but these were exceptional alkali soils.

From this study, Fred's limits of $pH$ 6.6 and 8.4 to 8.8 would seem to be too narrow. Different strains of *Azotobacter*, however, may show variations in the effect of reactions on growth, and since this strain was an especially vigorous one its limits might be expected to be wider.
SUMMARY

A vigorous strain of *Azotobacter chroococcum* was grown in solutions whose reactions were definitely determined by the hydrogen electrode. The nitrogen fixed in the solutions of each reaction was determined and the changes in reaction during incubation were measured.

It was found that the reaction of the solutions below $P_H$ 8.0 changed very little, those below $P_H$ 6.0 because no growth occurred, and those between 6.0 and 8.0 because the solution in this region was highly buffered.

Above $P_H$ 8.0 the reaction changed greatly, possibly due to incomplete reaction of the alkali at the time of titration, but more probably due to absorption of carbon dioxide by the strong alkali.

The amount of nitrogen fixed was not greatly affected by reactions between $P_H$ values of 6.2 and 8.8 although reactions around $P_H$ 7.0 and 8.0 seemed to be most favorable. Slight changes outside of these values caused an abrupt decrease in fixation.

LITERATURE CITED

1 Fred, E. B., and Davenport, A.

2 Gainey, P. L.

3 Gainey, P. L.

4 Waksman, S. A.