THE INFLUENCE OF ROENTGEN RAYS UPON THE NITROGEN FIXATION BY AZOTOBACTER

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INTRODUCTION

Nitrogen fixation by living organisms must be considered one of the fundamental life processes. The importance of investigations directed towards further elucidation of this little understood process need scarcely be emphasized. During the past four decades, well over a thousand published papers have resulted from the study of just one of these organisms, the free-living aerobic nitrogen-fixing bacterium Azotobacter.

Experiments with ionizing radiations have given valuable information in studies on the "sensitive volumes" associated with mutations in Drosophila (Haskins and Enzmann, 1936; Enzmann and Haskins, 1938), and on the mechanism of photosynthesis in Chlorella pyrenoidosa (Arnold, 1933); and experiments with a similar purpose have been made with low voltage cathode rays on Aspergillus (Whelden and Haskins, 1938; Buchwald and Whelden, 1939; Whelden et al., 1940). Work employing radiations in the study of the mechanism of nitrogen fixation by Azotobacter is very meager.

The influence of visible light upon the rate of nitrogen fixation has been noted by several workers, but no comprehensive study has been made. It has been stated that nitrogen fixation takes place in light as well as in darkness. Kayser (1920) tested the influence of visible light upon N-fixation by using differently colored glass containers as culture vessels. He reported that nitrogen fixation was small in violet-colored vessels and large in brown ones. Mannite as energy source was completely used up in 3 months at room temperature in brown, green, and black containers. The same author (Kayser, 1921) tested the influence of uranium salts (acetates and nitrates) upon N-fixation and found that the utilization of mannite as well as nitrogen fixation was increased thereby. With glucose as energy source the effect was even more pronounced. The addition of powdered radioactive salt to culture media (Kayser and Delaval, 1924) increased nitrogen fixation by as much as 75 per cent. Stoklasa (1920) experimented with potassium which emits β and γ rays, and agreed with others who had reported it to stimulate various life processes such as photosynthesis and embryonic development. He found that radium emanations at amounts of 80 to 150 ME (Millieinheiten) were not harmful to nitrogen fixation, and indeed even stimulated it.
The present investigations attempt to answer the following questions:
1. What functional relationship can be obtained between nitrogen fixation and graded x-ray doses?
2. Does exposure to x-rays affect respiration and nitrogen fixation in the same way and to the same degree?

**Material and Methods**

The three species of *Azotobacter* used in the present work: *Azotobacter chroococcum*, *A. agilis*, and *A. vinelandii* were originally obtained through the kindness of Dr. Dean Burk and have been cultured in our laboratory on agar slants from which inoculations were made to liquid cultures as required. The organisms were grown in liquid culture in 250 cc. Erlenmeyer flasks closed with perforated rubber stoppers holding the glass and rubber aerating systems. The rate of air flow could be regulated by a set screw on each valve. The rate of bubbling of air through the cultures, the temperature of the incubator, the composition of the medium, and the handling of the cultures were in accordance with the methods described by Burk (1930). Burk's method of culturing in gas wash-bottles was also used at first, but was abandoned since it was more difficult to handle and did not give any better results than the method here described.

*Azotobacter* cultures were incubated up to 24 hours, during which time the cell count rose to 30,000–150,000 cells per cubic millimeter, depending mainly on the rate of aeration.

All counts were made with a Neubauer hemocytometer after diluting the culture to an approximate cell count of 20,000–30,000 cells per cubic millimeter. A drop of acetic acid added to 20 cc. of culture medium shortly before counting stops all motility of the cells and facilitates counting by increasing their refractive index.

After removing the cultures from the aerating system they were tested for pH with brom thymol blue solution; for sugar content with Nessler solution; for freedom from contamination, by microscopic examination; for cell density by cell count or by centrifuging. Suitable samples of cultures were well shaken to insure even cell distribution and divided into two portions of 30 cc. each, one of which was x-rayed while the other was kept as a control. The x-raying was done in Harvard jars at 35 cm. target distance with a Coolidge x-ray machine, delivering 330 Roentgen units per minute at 168 kv. peak voltage and 10 milliamperes. No filters were used. The x-ray doses employed were 500 r., 1000 r., 3000 r., 3300 r., 5000 r., 10,000 r., and 20,000 r. Two controls were run simultaneously with each set of experiments: one unrayed culture and a H,O control.

Nitrogen fixation was measured by the method used and described by Burk in his experiments demonstrating N-fixation by manometric methods (1930a). Since previous workers have stated that the efficiency of N-fixation reaches a maximum at high partial pressures of gaseous nitrogen (Meyerhof and Burk, 1928; Burk, 1930b), we used a prepared gas mixture, containing about 1 per cent O₂.

In handling the Warburg manometers we generally followed the method employed by Burk to demonstrate nitrogen fixation manometrically, except for minor modifications.

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1 By a method described by Burk, 1930 a.
The gas mixture we used contains less oxygen than that recommended for maximal efficiency of nitrogen fixation by Burk, but has the advantage that the geometry of the apparatus permits a complete gas analysis.

The success of the experiment depended mainly on the accuracy of measuring small quantities of gas volumes. The greatest single source of error was the determination of the manometer reading corresponding to the starting point. This depends on temperature adjustments, degassing of liquids, and the gas consumption by the organisms. The first two factors tend to raise the manometer level, the last reduces it. Corrections are applied the following way. Temperature equilibration produces manometer reading changes which follow Newton's law and which under our experimental conditions are virtually completed in 15 minutes. The degassing of the liquids in the Warburg vessels, or more correctly the establishment of new partial pressures of gases in the manometers and corresponding changes in the proportion of dissolved gases, requires 30-60 minutes and proceeds nearly linearly with time in the final stage. The decrease in gas space due to respiration and nitrogen fixation takes place as soon as the vessels are filled and the stopcocks are closed. The corrections are determined by observations on control vessels containing H₂O instead of cultures. The importance of applying such corrections is shown by the fact that the establishment of new gas equilibria produces an average change in gas volume equal to about 20 per cent of the volume of the nitrogen removed during fixation.

The influence of the hydrostatic pressure of the manometer fluid upon the measurement of gas volumes was eliminated largely by adjusting the manometer levels so that they would be as far above the zero mark ( = 150 mm.) at the start of the experiment, as they would be below the zero mark at the end.

Most of the experiments were terminated about 4 hours after x-raying the material, in order to avoid the possible recovery due to new cell generations of Azotobacter. Furthermore, carrying the experiments to completion, i.e. until the cells had consumed all available oxygen, was found to add little to the results since about 65 per cent of the oxygen is consumed during the first 3 hours and the rate of oxygen consumption as well as the efficiency of nitrogen fixation declines considerably at very low oxygen pressures.

RESULTS

A. Respiration

The experiments summarized below were done under uniform standard conditions defined by:

<table>
<thead>
<tr>
<th>Condition</th>
<th>Specification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age of culture</td>
<td>20-24 hrs.</td>
</tr>
<tr>
<td>Cell count</td>
<td>30-200 thousand cells/cm³</td>
</tr>
<tr>
<td>Cell picture</td>
<td>free of contaminants</td>
</tr>
<tr>
<td>pH</td>
<td>7.0-7.3</td>
</tr>
<tr>
<td>Bath temperature</td>
<td>28.2 ± 0.02°C</td>
</tr>
<tr>
<td>Shaker speed</td>
<td>140 cycles/min.</td>
</tr>
<tr>
<td>Duration of run</td>
<td>3 hrs.</td>
</tr>
</tbody>
</table>

At the end of each run the remaining oxygen was removed with alkaline pyrogallol, by mixing the culture with 0.3 cc. of concentrated pyrogallic
acid and 0.3 cc. saturated solution of KOH. The rate of respiration of a normal unrayed culture of Azotobacter compared with that of cultures which had received a dose of 3300 r. is shown by Fig. 1. The vessels contained atmospheric air.

All curves made under the same conditions show a slight initial rise in the rate of respiration followed by a period of almost constant rate. When the same experiment is repeated under an atmosphere of 99 per cent N₂ and 1 per cent O₂ the initial rise in the rate of respiration is extremely brief and is quickly followed by a decline in respiration rate (Fig. 2). Very little difference has been found between x-rayed and untreated cells in the rate of oxygen consumption per cell per hour, within the radiation dosages used (0.278μ³ O₂/cell/hour for unrayed controls; 0.280μ³ O₂/cell/hour for x-rayed cells are average amounts). It may be concluded that respiration is not markedly affected by xraying the cells within the dosage range represented by the present experiments. A slight stimulation of respiration was often observed in x-rayed cultures, which is shown in the early periods in Figs. 1 and 2. It is noted that the final rate of oxygen consumption of x-rayed cells is slightly lower than that of the unrayed controls, although it was nearly identical at the start of the experiment. This is explained by the slower rate of cell division of x-rayed cells. Actual counts of x-rayed samples and control samples give figures which are commensurate with the measured differences in the rate of respiration.

B. Nitrogen Fixation

In contrast to respiration, nitrogen fixation is markedly affected by x-irradiation. This is shown in Table I. Column I indicates the treatment
of the culture, column II the total fall of the manometer level due to removal of oxygen as well as nitrogen by respiration and nitrogen fixation, as well as by KOH and pyrogallic acid. Column III shows the amount of oxygen and nitrogen removed by respiration and nitrogen fixation alone, column IV the amount of nitrogen fixed, and column V the amount of oxygen used up in respiration while fixation went on. All volumes are in cubic millimeters at normal temperature and pressure. The contents of the table are visualized easier by reference to the diagram (Fig. 3) drawn to scale, which also illustrates the method of calculating the efficiency of nitrogen fixation.

### TABLE I

<table>
<thead>
<tr>
<th>Treatment applied</th>
<th>I</th>
<th>II</th>
<th>III</th>
<th>IV</th>
<th>V</th>
<th>VI</th>
<th>VII</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>per cent</td>
</tr>
<tr>
<td>H₂O controls</td>
<td>163.3</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>107.8</td>
<td>8.75</td>
<td>146</td>
</tr>
<tr>
<td>0 r. controls</td>
<td>172.8 ± 0.40</td>
<td>117.3</td>
<td>9.5</td>
<td>107.8</td>
<td>8.70</td>
<td>74</td>
<td></td>
</tr>
<tr>
<td>500 r.</td>
<td>172.4 ± 0.41</td>
<td>118.2</td>
<td>9.4</td>
<td>108.8</td>
<td>8.0</td>
<td>71</td>
<td></td>
</tr>
<tr>
<td>1000 r.</td>
<td>171.3 ± 0.82</td>
<td>112.9</td>
<td>8.3</td>
<td>104.6</td>
<td>6.5</td>
<td>61</td>
<td></td>
</tr>
<tr>
<td>3000 r.</td>
<td>169.9 ± 0.34</td>
<td>111.7</td>
<td>6.9</td>
<td>104.8</td>
<td>6.5</td>
<td>61</td>
<td></td>
</tr>
<tr>
<td>5000 r.</td>
<td>167.5 ± 0.42</td>
<td>103.1</td>
<td>4.5</td>
<td>108.6</td>
<td>4.6</td>
<td>76</td>
<td></td>
</tr>
<tr>
<td>10000 r.</td>
<td>164.6</td>
<td>108.5</td>
<td>0.9</td>
<td>107.6</td>
<td>0.83</td>
<td>23</td>
<td></td>
</tr>
</tbody>
</table>

Beside each horizontal bar is indicated the nature of the experiment and the bar itself represents the fall of the manometer level during the experiments. The length of the bar marked H₂O indicates the number of cubic millimeters of O₂ and CO₂ removed by KOH and pyrogallic acid. This amount would have been removed from all vessels had they contained H₂O instead of living *Azotobacter*. The excess drop in culture vessels over that in H₂O containing vessels measures therefore the amount of nitrogen removed by *Azotobacter* from the gas mixture, or the amount of nitrogen fixed. Column IV of Table I as well as Fig. 3 show that unrayed cultures fix the largest amount of nitrogen, and that the amount of N₂ fixed decreases regularly with increasing x-ray dose.

The initial experiments (500 r.-5000 r.) showed that nitrogen fixation decreased linearly with increasing x-ray dose. One could therefore by extrapolation determine the x-ray dose which would abolish N-fixation completely. Fig. 4 shows that this dose should be about 11,000 r. Subsequent
determinations with very high x-ray doses (10,000 r., 20,000 r.) showed that the 100 per cent effective dose is higher than expected and that the decrease of N-fixation as a function of x-ray dose is not strictly linear.

C. Efficiency

The efficiency of nitrogen fixation is defined as the amount of nitrogen fixed divided by the amount of oxygen used up during the time when fixation took place.
Reference to the diagram (Fig. 3) shows that the true amount of O$_2$ used in respiration can be found by subtracting the amount of nitrogen fixed (striped bars) from the total decrease in gas space (indicated by bracket and legend), before KOH and pyrogallic acid were mixed with the cultures and the germs were killed. Table I (column VI) shows that the efficiency of fixation also decreases regularly with an increase of the x-ray dose.

It should be noted that several experiments were carried on until practically all oxygen was removed by respiration, and spilling of KOH plus pyrogallic acid produced no further fall. These control experiments agreed substantially with the ones detailed in the table, the total amounts of nitrogen fixed being but slightly higher.

**DISCUSSION**

It is generally thought that respiration as well as nitrogen fixation are chain reactions governed by enzyme systems. The system involved in N-fixation by *Azotobacter* has been discussed by Burk (1934). Several other schemes have been put forward, most of which involve the assumption that the energy derived from the respiration is used to drive the second mechanism namely nitrogen fixation. This may be represented as follows:

\[
\begin{align*}
\text{Respiration:} & \quad \text{sugar} + O_2 \rightarrow A \rightarrow B \rightarrow CO_2 + H_2O. \\
\text{N-fixation:} & \quad N + H_2O \rightarrow P \rightarrow Q \rightarrow \text{fixed N (protein)}. 
\end{align*}
\]

The present experiments indicate that the respiratory chain of events as a whole is not affected to any great extent by x-rays of the doses used. Nitrogen fixation however is affected and decreases in a regular fashion with increasing x-ray dose. It can therefore be stated with some assurance, that the two processes, respiration and N-fixation, can be dissociated to an extent depending on the x-ray dose. Such a dissociation of the two processes is not entirely new and may take place spontaneously; a considerable number of students have reported a sudden loss of the power of *Azotobacter* to fix nitrogen. The dissociation can also be brought about by offering to *Azotobacter* certain energy foods which support respiration more or less completely but on which the organism is unable to grow. (We shall report on these experiments in a later communication.)

Arnold (1933) reported a similar dissociation of respiration and photosynthesis, when *Chlorella* was irradiated with ultraviolet light.

**SUMMARY**

The influence of graded x-ray doses upon nitrogen fixation and respiration by *Azotobacter* was studied by means of the Warburg method. It was found that nitrogen fixation decreases approximately linearly with in-
creasing x-ray doses. Respiration in contrast is affected only indirectly through some inhibition of cell multiplication. Small doses of x-ray often produce a slight and transient increase in the rate of oxygen uptake.

LITERATURE CITED


