1928

I. Some effects of manganese sulfate and manganese chloride on nitrification; II. The isolation of some nitrifying organisms

Daniel Hans Nelson  
Iowa State College

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UMI
I. SOME EFFECTS OF MANGANESE SULFATE AND MANGANESE CHLORIDE ON NITRIFICATION

II. THE ISOLATION OF SOME NITRIFYING ORGANISMS

BY

Daniel Hans Utlen

A Thesis submitted to the Graduate Faculty for the Degree of

DOCTOR OF PHILOSOPHY

Major subject Soil Bacteriology

Approved

Signature was redacted for privacy.
In charge of Major work
Signature was redacted for privacy.
Head of Major Department
Signature was redacted for privacy.
Dean of Graduate College

Iowa State College
1928
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I. SOME EFFECTS OF MANGANESE SULFATE AND MANGANESE CHLORIDE ON NITRIFICATION

INTRODUCTION

The relationships of manganese compounds to plant growth have been studied quite extensively by several investigators during the last thirty-five years. While it has not been absolutely proved that manganese is an essential element, it is well established that when present in low concentrations it may exert a beneficial influence on some plants. It is not certain by any means that all plants require the same elements; and indeed it seems very probable that an element may be absolutely essential for one plant and quite unnecessary for another. While it has been shown that some plants are benefitted by the presence of manganese, it is not known how it exerts its favorable action although it is usually believed that it assists the oxidative processes in metabolism. Whether manganese acts as an oxidase or is a constituent of an organic oxidase, formed by the plant, is not entirely clear; but in either case the effects would be the same.

The question then arises do manganese compounds
affect microorganisms in the same way that they affect the higher plants. Up to the present time there have been insufficient studies along this line to warrant a conclusion. However, since most bacteria seem to be closely related to higher plants, it is probable that the effects would be much the same.

One physiological group of soil organisms are able to obtain energy for life processes by oxidising ammonium compounds or nitrites. The process is spoken of as nitrification and the organisms are called nitrifiers. If it is true that manganese stimulates oxidative processes in higher plants, would it not be possible and even probable that it would stimulate the oxidative processes of these bacteria? It was with a hope of throwing more light on this subject that this work was planned.

REVIEW OF LITERATURE

Most of the work dealing with the effects of manganese on living organisms has been carried out with higher plants. Numerous pot and field tests have been made using varying amounts of different manganese compounds mixed with the soil and growing plants of
various kinds. The dry weight of the crop, the weight of grain or both have been taken as a measure of the effectiveness of the treatments. This work has been well reviewed by Olrau (9) and Branchley (1) and extensive bibliographies given.

Skinner and Sullivan (13) found that manganese salts increased the oxidizing power of plant roots in poor soils, and stimulated growth. In rich soils oxidation was increased but plant growth was retarded the plants showing indications of excessive oxidation. When 50 pounds per acre were added the oxidizing power of the soil was decreased and the crop yield lowered. The latter result they suggest was probably due to the fact that the soils were acid in character.

Skinner and Reed (12) studied the effects of manganese sulfate on an acid soil using 50 pounds of the salt per acre. They found that the oxidative power of the soil was increased only when the soil was neutralized with calcium carbonate before the manganese was added. The yields of wheat, rye, corn and cowpeas were increased but there was no effect on the potatoes. When manganese was added to the soil without neutralising the acidity, the oxidative power was decreased as well as the yields.
Schreiner and Sullivan (11) found that oxidation in soils is increased by salts of manganese, iron, aluminum, calcium and magnesium, especially in the presence of hydroxy acids such as citric, tartaric, malic, glycolic and their salts. The best oxidation was obtained by the addition of manganese. The authors believe this property of manganese salts is sufficient to account for the stimulating effects it often shows on crop growth.

Kelley (5) in a study of Hawaiian soils found that the various species of bacteria considerably vary in their behavior toward manganese just as is the case with different higher plants. Nitrification took place more rapidly in manganiferous soils than in normal soils, while ammonification was about the same in both. He believes that the aeration was quite largely responsible for this as the normal soil used was more compact and hence not so well aerated. Experiments carried out later (6) confirmed these results.

Brown and Hinges (2) studied the effects of manganese sulfate, manganese chloride, manganese nitrate, and manganese oxid on ammonification and nitrification in Carrington loam. Manganese chloride in amounts of 100 pounds to 200 pounds per acre increased nitrification while greater amounts depressed it. Manganese sulfate in
amounts up to 100 pounds per acre increased nitrification but larger applications up to 200 pounds per acre had no influence. Manganese nitrate in all amounts used depressed nitrification as did also manganese oxid.

Montanari (8) using 50 grams of soil per petri dish treated with varying amounts of manganese oxid, manganese sulfate, or manganese carbonate found stimulating effects on nitrification shown by all these compounds in the concentrations used. The amounts of manganese used, however, did not exceed 0.5 gram per 50 grams of soil.

Leoncini (7) conducted experiments with lime soils, clay soils and humus soils to determine the effects of manganese dioxide and natural manganese hydrate on the nitrification of ammonium sulfate. In amounts up to 0.184 percent these compounds stimulated nitrification but in larger amounts nitrification was retarded or inhibited.

Greaves, Carter and Goldthorpe (4) tested the effects of several salts of manganese on nitrification in a calcareous loam. Every salt tested showed stimulation in one or more of the concentrations used and all became toxic at the higher concentrations.

Deatrick (3) found that 10 to 20 p.p.m. of manganese sulfate did not affect the nitrifying power of
the soil. Larger amounts of manganese checked nitrification.

Pietruszczynski (10) studied the influence of manganese salts on nitrification. All were found to increase nitrification when they were used in low concentrations. Of the salts tested, manganese sulfate had the greatest stimulating effect followed in order by manganese chloride and manganese carbonate. Soil and liquid cultures showed about the same results.

It is clear from the above brief review that manganese compounds influence oxidative processes in the soil and that the nitrifying bacteria are stimulated by certain concentrations of manganese compounds.

EXPERIMENTAL

It is clear from an examination of the papers dealing with the influence of manganese salts on nitrification that the negative ion was not often been adequately considered. The salt or salts have in most cases been added to the soil or solution and the amount of oxidized nitrogen determined after the incubation period. It is not clear from such data whether the effects noted were due to the manganese, the negative ion, or to both; and the amount of the effect due to each is entirely
unknown. Of course it is impossible to add manganese to a soil, except as the metal, without adding at the same time some other constituent. The fault is not so much with the method as with the interpretation of the results.

Greaves (4) studied several salts in series having common anions all used in molal equivalents. Comparisons were then made of all chlorides, all nitrates, etc., as a measure of the effects produced by the various positive ions. This is perhaps the most satisfactory method for a study of this kind so far employed but it required an enormous amount of work. The author wished to get some idea of the effects due to the negative ions but could not do the extensive work necessary by this method owing to a lack of time. It was thought advisable to supply an equivalent concentration of the negative ions by using a mixture of salts. In this way each positive ion would be present in small concentration, the amount diminishing with the number of salts used in the mixture. Then by using positive ions common in the soil the effects of the small amounts of each positive ion added would be relatively slight while the negative ion concentration would be equal to that of the soil treated with the manganese salt. With this purpose in mind, sulfate and chloride solutions were prepared which were equivalent
in sulfate and chloride content per c.c. to the manganese sulfate and manganese chloride solutions used. The sulfate solution was made up in such a way that an equal proportion of the sulfate ion was supplied by each of the following salts: MgSO₄, K₂SO₄, Na₂SO₄, and Fe₂(SO₄)₃. This solution will be called the sulfate solution in the tables. A similar chloride solution was prepared whose concentration of chloride ions per c.c. was equal to that of the manganese chloride solution used. The following salts were used, each supplying an equal proportion of the chloride ions: KCl, NaCl, MgCl₂, CaCl₂ and FeCl₃. This solution will be called the chloride solution in the tables.

By using these solutions soils could be treated with concentrations of SO₄ ions and Cl ions equal to those added with the manganese treatments. The positive ions would each be increased very slightly and since common soil ions were used it was believed that their effects would be at a minimum. However, it should be emphasized that a comparison is being made between manganese and a number of positive ions together since these are the only conditions not kept as nearly alike as possible. If manganese does not stimulate nitrification compared in this way, it will at least be safe to conclude that its...
use for stimulating purposes would be undesirable, for the salts compared are cheaper and more easily available than manganese salts.

**Materials and Methods**

This work was done in the summer and fall of 1927. The soils used were Carrington loam and Webster loam taken from the Agronomy Farm of the Iowa Agricultural Experiment Station. The soils were air-dried, sieved and stored in earthenware jars.

The tumbler method was used to study nitrification, 100 grams of soil being used for each sample. Dried blood containing 108.5 mgm. nitrogen per gram was added at the rate of 1 percent and ammonium sulfate sufficient to supply 30 mgm. nitrogen per 100 gram of soil was used. The samples were all incubated at room temperature in the dark in duplicate. The moisture content of the soils was made up to the optimum at the beginning of the experiment and once each week during incubation by weighing the samples and making up the loss in weight with distilled water.

The phenoldisulphonic acid method was employed for all nitrate determinations, the chlorides being removed with silver sulfate in the chloride series.
Table 1 shows the amounts of various constituents in the soils used. These data show the condition of the soils from the standpoint of those factors most essential in this study. It should be noted that the Webster soil is much higher in total nitrogen, is basic in reaction, and contains slightly more nitrate nitrogen and total manganese. The Carrington, on the other hand, is comparatively low in total nitrogen and shows an acid reaction. It was decided to test this soil in its natural condition as well as with the acidity neutralized with CaCO₃.

Results

The results of the nitrification tests are shown in tables 2 to 11 inclusive.

With Manganese Sulfate

In these tables the first column gives the various treatments of manganese as milligrams of manganese supplied as manganese sulfate or manganese chloride. The other vertical columns show milligrams of nitrate nitrogen per 100 grams of soil at the end of the incubation period, and the pH values of the respective soils at the time of analysis. When the sulfate solution was used the soils received varying amounts of the solution sufficient to...
TABLE I

Various Constituents per 100 Grams of dry soil.

<table>
<thead>
<tr>
<th></th>
<th>pH</th>
<th>Moisture</th>
<th>Total</th>
<th>Nitrate</th>
<th>Ammonium</th>
<th>Nitrogen</th>
<th>Magnesium</th>
<th>Manganese</th>
<th>Manganese</th>
<th>Manganese</th>
</tr>
</thead>
<tbody>
<tr>
<td>Webster loam</td>
<td>8.1</td>
<td>5.20</td>
<td>364.0</td>
<td>0.81</td>
<td>1.71</td>
<td>trace</td>
<td>1.12</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Carrington loam</td>
<td>6.5</td>
<td>3.02</td>
<td>252.0</td>
<td>0.15</td>
<td>1.54</td>
<td>trace</td>
<td>1.00</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
make the sulfate ions equal to those in the soils treated with manganese sulfate. In table 2, for example, the soil receiving .01 mgm. of manganese as manganese sulfate contained 125.7 mgm. nitrate nitrogen while that one receiving a similar $SO_4$ concentration as a sulfate mixture contained 137.8 mgm. nitrate nitrogen. This arrangement was chosen to facilitate the examination of corresponding treatments in parallel columns.

An examination of table 2, shows that in the case of the dried blood all the added nitrogen was nitrified and hence a comparison of rates of nitrification would be of little value. There is a lower nitrification of the nitrogen in the presence of manganese sulfate than in the presence of an equivalent amount of sulfates from the sulfate solution. In only two cases is more nitrogen oxidized in the presence of manganese than in similar untreated soil while with the sulfate solution there was a higher oxidation in all but one case and then it was equal to that in the untreated soil.

In every case more ammonium sulfate was nitrified in the presence of manganese sulfate than in untreated soil but the same was true in the presence of the sulfate solution in all but two cases. With 5 and 10 mgm. of manganese respectively there was a decided
TABLE 2

Effects of Manganese Sulfate and Various Other Sulfates on Nitrification in Webster Loam.

<table>
<thead>
<tr>
<th>Mgm. of Mn.</th>
<th>Mgm. of As MnSO$_4$</th>
<th>Nitrate nitrogen</th>
<th>pH</th>
<th>Mgm. of MnSO$_4$</th>
<th>Mgm. of As MnSO$_4$</th>
<th>Nitrate nitrogen</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Check</td>
<td>126.8</td>
<td>7.4</td>
<td></td>
<td>27.3</td>
<td>7.7</td>
<td>27.3</td>
<td>7.7</td>
</tr>
<tr>
<td>0.01</td>
<td>125.7</td>
<td>7.4</td>
<td></td>
<td>29.2</td>
<td>7.8</td>
<td>30.7</td>
<td>7.7</td>
</tr>
<tr>
<td>0.10</td>
<td>106.1</td>
<td>7.5</td>
<td></td>
<td>30.8</td>
<td>7.7</td>
<td>31.7</td>
<td>7.7</td>
</tr>
<tr>
<td>1.00</td>
<td>137.8</td>
<td>7.4</td>
<td></td>
<td>29.8</td>
<td>7.7</td>
<td>30.7</td>
<td>7.8</td>
</tr>
<tr>
<td>5.00</td>
<td>129.3</td>
<td>7.4</td>
<td></td>
<td>31.1</td>
<td>7.8</td>
<td>28.0</td>
<td>7.7</td>
</tr>
<tr>
<td>10.00</td>
<td>117.0</td>
<td>7.6</td>
<td></td>
<td>30.4</td>
<td>7.9</td>
<td>24.8</td>
<td>7.7</td>
</tr>
<tr>
<td>25.00</td>
<td>119.5</td>
<td>7.6</td>
<td></td>
<td>28.7</td>
<td>8.0</td>
<td>28.7</td>
<td>7.8</td>
</tr>
</tbody>
</table>

* This solution is made up of MgSO$_4$, K$_2$SO$_4$, Na$_2$SO$_4$ and Fe$_2$(SO)$_3$ such that an equal amount of SO$_4$ ions is supplied by each salt. This solution has the same SO$_4$ concentration per c.c. as the manganese solution used.
stimulation of nitrification by the manganese sulfate but in lower concentrations the sulfate solution brought about a higher oxidation.

Tables 3 and 4 show the effects of reaction on nitrification. Only 69.6 mgm. of nitrogen were nitrified in the natural soil while in the soil treated with one-half gram of CaCO₃ all of the added nitrogen was nitrified and 15.4 mgm. of the soil's own nitrogen besides. There was a slight stimulation of nitrification of dried blood where 0.1 mgm. of manganese was added to the unlimed soil; otherwise, all manganese and sulfate solution treatments resulted in depressions. In the case of ammonium sulfate up to 10 mgm. of manganese stimulated the oxidation in the acid soil, but the sulfate solution gave a much greater corresponding stimulation in every case. For the limed soil 1.0 mgm. of manganese stimulated nitrification of the dried blood while all other treatments had no effect or depressed oxidation.

There is no regularity in these results owing perhaps to the long incubation period chosen. Two of the sets were repeated using a 14 day incubation period. The results are given in tables 5 and 6.

Table 5 shows that there was no stimulation in the nitrification of dried blood and but very slight
**TABLE 3**

Effects of Manganese Sulfate and Various Other Sulfates on Nitrification in Carrington Loam.

<table>
<thead>
<tr>
<th>Mgm. of Mn</th>
<th>Mgm. of nitrate</th>
<th>With MnSO₄ solution(s)</th>
<th>With MnSO₄</th>
<th>With sulfate</th>
<th>With sulfate</th>
<th>Check</th>
<th>0.01</th>
<th>0.10</th>
<th>1.00</th>
<th>5.00</th>
<th>10.00</th>
<th>25.00</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>With Dried Blood</td>
<td>With Ammonium Sulfate</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MgSO₄</td>
<td>K₂SO₄</td>
<td>Na₂SO₄</td>
<td>Fe₂(SO₄)₃</td>
<td>MgSO₄</td>
<td>K₂SO₄</td>
<td>Na₂SO₄</td>
<td>Fe₂(SO₄)₃</td>
<td>MgSO₄</td>
<td>K₂SO₄</td>
<td>Na₂SO₄</td>
<td>Fe₂(SO₄)₃</td>
<td>MgSO₄</td>
</tr>
<tr>
<td>0.0</td>
<td>5.2</td>
<td>69.6</td>
<td>5.2</td>
<td>5.75</td>
<td>5.3</td>
<td>5.7</td>
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<td>15.3</td>
<td>5.2</td>
<td>19.5</td>
<td>5.3</td>
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<tr>
<td>0.1</td>
<td>5.5</td>
<td>57.4</td>
<td>5.4</td>
<td>7.42</td>
<td>5.3</td>
<td>15.3</td>
<td>5.2</td>
<td>19.5</td>
<td>5.3</td>
<td>22.8</td>
<td>5.1</td>
<td>5.3</td>
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<tr>
<td>1.0</td>
<td>5.5</td>
<td>74.2</td>
<td>5.3</td>
<td>7.42</td>
<td>5.3</td>
<td>19.5</td>
<td>5.3</td>
<td>22.8</td>
<td>5.1</td>
<td>22.8</td>
<td>5.1</td>
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<td>5.0</td>
<td>5.5</td>
<td>61.9</td>
<td>5.3</td>
<td>8.96</td>
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<td>25.0</td>
<td>6.2</td>
<td>49.8</td>
<td>5.1</td>
<td>5.97</td>
<td>5.6</td>
<td>25.1</td>
<td>5.1</td>
<td>15.6</td>
<td>5.5</td>
<td>15.6</td>
<td>5.2</td>
<td>5.3</td>
</tr>
</tbody>
</table>

*This solution is made up of MgSO₄, K₂SO₄, Na₂SO₄ and Fe₂(SO₄)₃ such that an equal amount of SO₄ ions is supplied by each salt. This solution has the same SO₄ concentration per c.c. as the manganese solution used.*
TABLE 4

Effects of Various Amounts of Manganese Sulfate and Other Sulfates on Nitrification in Carrington Loam Neutralized with CaCO₃

<table>
<thead>
<tr>
<th>Mgm. of MnSO₄: nitrogen</th>
<th>pH</th>
<th>Mgm. of MnSO₄: nitrogen</th>
<th>pH</th>
<th>Mgm. of MnSO₄: nitrogen</th>
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<tr>
<td>check</td>
<td></td>
<td>123.9</td>
<td>6.7</td>
<td>123.9</td>
<td>6.7</td>
</tr>
<tr>
<td>0.01</td>
<td></td>
<td>65.9</td>
<td>6.5</td>
<td>100.1</td>
<td>6.5</td>
</tr>
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<td>0.10</td>
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<td>88.0</td>
<td>6.2</td>
</tr>
</tbody>
</table>

* This solution is made up of MgSO₄, K₂SO₄, Na₂SO₄ and Fe₂(SO₄)₃ such that an equal amount of SO₄ ions is supplied by each salt. This solution has the same SO₄ concentration per c.c. as the manganese solution used.
TABLE 5
Effects of Manganese Sulfate and Various Other Sulfates on Nitrification in Webster Loam.

<table>
<thead>
<tr>
<th>Mgm. of MnSO₄</th>
<th>Mgm. of Kgrn. of with sulfur</th>
<th>Mgm. of MnSO₄</th>
<th>Mgm. of with sulfate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>with sulfate solutions</td>
<td></td>
<td>with sulfate solutions</td>
</tr>
<tr>
<td>Mgm. of Mn.</td>
<td>nitrate</td>
<td>Mgm. of nitrate</td>
<td>pH</td>
</tr>
<tr>
<td>As MnSO₄</td>
<td>nitrogen</td>
<td>pH</td>
<td>nitrogen</td>
</tr>
<tr>
<td>Check</td>
<td>32.3</td>
<td>6.8</td>
<td>32.3</td>
</tr>
<tr>
<td>0.01</td>
<td>29.1</td>
<td>6.8</td>
<td>40.4</td>
</tr>
<tr>
<td>0.10</td>
<td>32.1</td>
<td>6.9</td>
<td>38.4</td>
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<td>34.3</td>
</tr>
<tr>
<td>25.00</td>
<td>30.4</td>
<td>7.2</td>
<td>33.2</td>
</tr>
</tbody>
</table>

* This solution is made up of MgSO₄, K₂SO₄, Na₂SO₄ and Fe₂(SO)₃ such that an equal amount of SO₄ ions is supplied by each salt. This solution has the same SO₄ concentration per c.c. as the manganese solution used.
TABLE 6

Effects of Manganese Sulfate and Various Other Sulfates on Nitrification in Carrington Loam, Neutralized With CaCO₄.

<table>
<thead>
<tr>
<th>Mgm. of Mn as MnSO₄</th>
<th>Mgm. of nitrate</th>
<th>pH</th>
<th>Mgm. of Mn as MnSO₄</th>
<th>Mgm. of nitrate</th>
<th>pH</th>
<th>Mgm. of Mn as MnSO₄</th>
<th>Mgm. of nitrate</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Check</td>
<td>36.7</td>
<td>6.7</td>
<td>36.7</td>
<td>6.9</td>
<td>17.0</td>
<td>7.0</td>
<td>17.0</td>
<td>7.4</td>
</tr>
<tr>
<td>0.01</td>
<td>33.5</td>
<td>6.8</td>
<td>25.0</td>
<td>7.0</td>
<td>17.8</td>
<td>7.1</td>
<td>17.0</td>
<td>7.4</td>
</tr>
<tr>
<td>0.10</td>
<td>39.2</td>
<td>6.8</td>
<td>37.9</td>
<td>6.8</td>
<td>17.4</td>
<td>7.4</td>
<td>19.1</td>
<td>7.3</td>
</tr>
<tr>
<td>1.00</td>
<td>39.4</td>
<td>6.8</td>
<td>37.9</td>
<td>6.8</td>
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<td>7.3</td>
</tr>
<tr>
<td>5.00</td>
<td>44.5</td>
<td>6.9</td>
<td>30.0</td>
<td>6.9</td>
<td>16.1</td>
<td>7.5</td>
<td>19.8</td>
<td>7.3</td>
</tr>
<tr>
<td>10.00</td>
<td>39.9</td>
<td>7.1</td>
<td>30.3</td>
<td>6.9</td>
<td>14.5</td>
<td>7.6</td>
<td>19.2</td>
<td>7.3</td>
</tr>
<tr>
<td>25.00</td>
<td>29.1</td>
<td>7.1</td>
<td>30.3</td>
<td>7.1</td>
<td>16.4</td>
<td>7.6</td>
<td>19.6</td>
<td>7.2</td>
</tr>
<tr>
<td>50.00</td>
<td>19.7</td>
<td>7.3</td>
<td>19.6</td>
<td>7.1</td>
<td>15.7</td>
<td>7.7</td>
<td>18.4</td>
<td>7.2</td>
</tr>
</tbody>
</table>

* This solution is made up of MgSO₄, K₂SO₄, Na₂SO₄ and Fe₅(SO)₃ such that an equal amount of SO₄ ions is supplied by each salt. This solution has the same SO₄ concentration per c.c. as the manganese solution used.
stimulation in the nitrification of ammonium sulfate by the various manganese treatments on the Webster loam. It should be noted too, that even 50 mgm. of manganese as manganese sulfate did not stop nitrification. The sulfate solution was more stimulating than manganese sulfate in every case when dried blood was used and in all but three cases where ammonium sulfate was employed. In these exceptional cases the differences were very small.

In the Carrington loam, table 6 where CaCO₃ had been added 0.1, 1.0 and 5.0 mgm. of manganese as manganese sulfate stimulated the nitrification of dried blood and in every case more nitrogen was oxidized than in the soils treated with an equivalent of the sulfate solution. With ammonium sulfate the case was different; here there was a slight stimulation in nitrification where .01 mgm. or 1.0 mgm. of manganese was added but the sulfate solution showed a stimulation in all but one case. The higher concentrations of manganese did not reduce nitrification more than 50 percent in the case of the dried blood and only about 4 percent with ammonium sulfate. This is quite remarkable considering that the application was equivalent to about two and one-half tons of manganese sulfate per acre or an amount sufficient to give the soil a content of 0.05 percent manganese.
Sand and Solution Tests

With Sand

50 gram portions of well washed sand were weighed into tumblers and treated with a nutrient solution containing per liter K$_2$HPO$_4$, 1 gm; NaCl, 2 gms; K$_2$SO$_4$, 0.5 gm; Fe$_2$SO$_4$, trace; and sufficient ammonium sulfate to supply 17.2 mgm. nitrogen per sample. The tumblers were incubated for twenty-eight days at room temperature after receiving 5 c.c. of a soil suspension each. The nitrates were determined in the usual way and the results are given in table 7.

A glance at this table shows that manganese sulfate had a depressing effect but this was not regular in nature nor in proportion to the concentration of manganese ions present. Unfortunately the series treated with the sulfate solution was lost and not repeated for lack of time. However, there was so much sulfate in the solution that a small addition would probably not have increased the nitrification.

With Solution

A solution was prepared having the same constituents as that used with sand but having only 15.0 mgm. of nitrogen as ammonium sulfate per 100 c.c. of solution.
<table>
<thead>
<tr>
<th>Mgm. manganese as MnSO₄</th>
<th>Check: 0.005: 0.05: 0.50: 2.50: 5.00: 12.50</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nitrate Nitrate in Mgm.</td>
<td>: 5.92: 3.40: 3.05: 3.25: 5.64: 3.66: 2.14</td>
</tr>
</tbody>
</table>

TABLE 7

Influence of MnSO₄ on Nitrification of Ammonium Sulfate in Sand.
One hundred c.c. portions were placed in flasks and sterilized. An excess of CaCO₃ was then added and 5 c.c. of a soil suspension was introduced into each flask. Manganese sulfate was added to one series in varying amounts and the sulfate solution to another. After 28 days the cultures were analysed for nitrates. The results are given in table 8.

From this table it is clear that a slight stimulation occurred with all but the highest amounts of sulfate manganese and even here, there was no depression. However, only in the case of 0.1 mgm. of manganese did the manganese treatments give a higher result than the corresponding sulfate solution treatment. In all other cases the sulfate solution was more stimulating. With the solution tests the manganese of the soil is practically eliminated as a factor but the results are similar to those obtained with the soil cultures.

Results of the Tests With Manganese Chloride

These tests were similar to those carried out with the sulfate. Table 9 shows the effects of manganese chloride and various other chlorides on the nitrification of dried blood and ammonium sulfate in the Webster loam.
### TABLE 8
Effects of Manganese Sulfate on Nitrification in Solution.

<table>
<thead>
<tr>
<th>Mgm. of Mn as MnSO₄</th>
<th>Mgm. of nitrate</th>
<th>Mgm. of nitrate</th>
<th>Mgm. of nitrogen</th>
</tr>
</thead>
<tbody>
<tr>
<td>Check</td>
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<td>11.57</td>
<td>11.57</td>
</tr>
<tr>
<td>0.01</td>
<td>11.78</td>
<td>12.76</td>
<td></td>
</tr>
<tr>
<td>0.10</td>
<td>11.92</td>
<td>11.08</td>
<td></td>
</tr>
<tr>
<td>1.00</td>
<td>11.85</td>
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</tr>
<tr>
<td>10.00</td>
<td>11.54</td>
<td>13.04</td>
<td></td>
</tr>
</tbody>
</table>

* This solution is made up of MgSO₄, K₂SO₄, Na₂SO₄ and Fe₂(SO₄)₃ such that an equal amount of SO₄ ions is supplied by each salt. This solution has the same SO₄ concentration per c.c. as the manganese solution used.
TABLE 9

Effects of Manganese Chloride and Other Chlorides on Nitrification in Webster Loam.

<table>
<thead>
<tr>
<th>Mgm. of MnCl₂</th>
<th>Mgm. of KCl</th>
<th>Mgm. of NaCl</th>
<th>Mgm. of CaCl₂</th>
<th>Mgm. of FeCl₃</th>
<th>Mgm. of MgCl₂</th>
<th>Check</th>
<th>0.01</th>
<th>0.10</th>
<th>1.00</th>
<th>5.00</th>
<th>10.00</th>
<th>25.00</th>
<th>50.00</th>
</tr>
</thead>
<tbody>
<tr>
<td>13.4</td>
<td>13.4</td>
<td>13.4</td>
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<td>7.9</td>
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<td>No pH</td>
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<td>9.5</td>
<td>9.5</td>
<td>9.5</td>
<td>9.5</td>
<td>9.5</td>
<td>9.5</td>
</tr>
<tr>
<td>12.5</td>
<td>18.3</td>
<td>18.3</td>
<td>7.9</td>
<td>7.9</td>
<td>8.5</td>
<td>values</td>
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<td>7.1</td>
<td>7.1</td>
<td>7.1</td>
<td>7.1</td>
<td>7.1</td>
<td>7.1</td>
</tr>
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<td>16.6</td>
<td>8.0</td>
<td>8.0</td>
<td>11.1</td>
<td>dcter-</td>
<td>9.8</td>
<td>9.8</td>
<td>9.8</td>
<td>9.8</td>
<td>9.8</td>
<td>9.8</td>
<td>9.8</td>
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<td>17.4</td>
<td>17.4</td>
<td>7.8</td>
<td>7.8</td>
<td>7.7</td>
<td>mined</td>
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<td>8.5</td>
<td>8.5</td>
<td>8.5</td>
<td>8.5</td>
<td>8.5</td>
<td>8.5</td>
</tr>
<tr>
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<td>16.1</td>
<td>16.1</td>
<td>8.0</td>
<td>8.0</td>
<td>8.4</td>
<td>dcter-</td>
<td>9.3</td>
<td>9.3</td>
<td>9.3</td>
<td>9.3</td>
<td>9.3</td>
<td>9.3</td>
<td>9.3</td>
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<td>7.8</td>
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<td>mined</td>
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<td>8.9</td>
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<td>8.9</td>
<td>8.9</td>
<td>8.9</td>
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<td>10.0</td>
<td>8.0</td>
<td>8.0</td>
<td>5.0</td>
<td>5.0</td>
<td>5.0</td>
<td>5.0</td>
<td>5.0</td>
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<td>2.6</td>
<td>2.6</td>
<td>2.6</td>
<td>2.6</td>
<td>2.6</td>
</tr>
</tbody>
</table>

* This solution is made up of MnCl₂, KCl, NaCl, CaCl₂, and FeCl₃ such that an equal amount of chloride ion is supplied by each salt. This solution has the same Cl concentration per c.c. as the manganese solution used.
Comparing these results with those reported in table 2, it is quite noticeable that the total amounts of nitrogen nitrified were much smaller with the chlorides than with the sulfates. This cannot be due to the chlorides, however, for the nitrate nitrogen accumulation in the untreated soil was correspondingly low. The only factor which can be suggested to account for this is temperature. This work was done in December while the previous work was done in September. Room temperature was used in both cases and there was probably a difference of 3-5 degrees.

In every case where dried blood was used the chloride solution showed a greater stimulation than the manganese chloride. Only where 1.0 mgm. of manganese as the chloride was added, was the nitrification equal to that in the control sample, while the chloride solution brought about stimulating effects at every concentration. There was no regularity in the stimulation, however, and no given concentration was superior in all cases.

The results with ammonium sulfate were more nearly uniform for both the manganese chloride and the chloride solution. There was a slight stimulation with 0.01 mgm. of manganese. The chloride solution showed the greatest stimulation with an amount equivalent to 25 mgm.
of manganese. It is noticeable that even the highest applications did not reduce the nitrifying power more than 10 to 15 percent.

With the Carrington loan, the unlimed soil was incubated 20 days. The results are shown in table 10. This table shows that there was no stimulation in any case where dried blood was used either with the manganese chloride or the chloride solution. At the higher concentrations the manganese was more toxic than the corresponding concentrations of chloride solution.

With ammonium sulfate there was a slight stimulation with .01 and 0.1 mgm. of manganese chloride, but with higher concentrations the toxicity is very marked practically stopping nitrification at 50 mgm. manganese. With the chloride solution the stimulation with the lower concentrations was more marked than with the manganese chloride; and with the higher concentrations, less toxic.

Table 11 shows the effects of lime. While there was no stimulation in the nitrification of the dried blood with the manganese chloride the toxic effects of the higher concentrations were markedly reduced. The chloride solution showed a slight stimulation at low concentrations, (.01 mgm.) and was less toxic at the higher concentrations.

With ammonium sulfate there was a marked
TABLE 10

Effects of Manganese Chloride and Various Other Chlorides of Nitrification in Carrington Loam.

<table>
<thead>
<tr>
<th>Mgm. of MnCl₂</th>
<th>Mgm. of Nitrate</th>
<th>Mn. as MnSO₄</th>
<th>Nitrogen</th>
<th>pH</th>
<th>Mgm. of Nitrate</th>
<th>Mn. as MnSO₄</th>
<th>Nitrogen</th>
<th>pH</th>
</tr>
</thead>
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<tr>
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<td>5.1</td>
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<td>5.2</td>
<td>11.8</td>
<td>5.4</td>
<td>11.8</td>
<td>5.4</td>
</tr>
<tr>
<td>0.01</td>
<td>49.4</td>
<td>5.1</td>
<td>45.5</td>
<td>5.3</td>
<td>12.3</td>
<td>5.3</td>
<td>12.5</td>
<td>5.4</td>
</tr>
<tr>
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<td>47.1</td>
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<td>12.1</td>
<td>5.3</td>
<td>12.9</td>
<td>5.3</td>
</tr>
<tr>
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<td>45.5</td>
<td>5.1</td>
<td>47.7</td>
<td>5.2</td>
<td>10.9</td>
<td>5.4</td>
<td>12.1</td>
<td>5.4</td>
</tr>
<tr>
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<td>42.7</td>
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<td>46.6</td>
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<td>10.1</td>
<td>5.4</td>
<td>11.9</td>
<td>5.3</td>
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<td>10.00</td>
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<td>40.0</td>
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<td>5.6</td>
<td>11.8</td>
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<tr>
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<td>23.3</td>
<td>6.1</td>
<td>38.5</td>
<td>5.4</td>
<td>3.9</td>
<td>5.8</td>
<td>10.0</td>
<td>5.4</td>
</tr>
<tr>
<td>50.00</td>
<td>16.0</td>
<td>6.4</td>
<td>23.2</td>
<td>5.9</td>
<td>0.9</td>
<td>6.0</td>
<td>5.2</td>
<td>5.7</td>
</tr>
</tbody>
</table>

* This solution is made up of MnCl₂, KCl, NaCl, CaCl₂, and FeCl₃ such that an equal amount of chloride ion is supplied by each salt. This solution has the same Cl concentration per c.c. as the manganese solution used.
<table>
<thead>
<tr>
<th>Mgm. of MnCl₂</th>
<th>Mgm. of MnSO₄</th>
<th>Mgm. of Mn, as Mn₂₃</th>
<th>With chloride</th>
<th>With chloride</th>
</tr>
</thead>
<tbody>
<tr>
<td>Check</td>
<td>52.4</td>
<td>7.2</td>
<td>32.4</td>
<td>7.1</td>
</tr>
<tr>
<td>0.01</td>
<td>32.8</td>
<td>7.1</td>
<td>38.7</td>
<td>7.1</td>
</tr>
<tr>
<td>0.10</td>
<td>31.8</td>
<td>7.1</td>
<td>31.2</td>
<td>7.2</td>
</tr>
<tr>
<td>1.00</td>
<td>32.8</td>
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<td>31.9</td>
<td>7.2</td>
</tr>
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<td>5.00</td>
<td>28.6</td>
<td>7.0</td>
<td>32.2</td>
<td>7.3</td>
</tr>
<tr>
<td>10.00</td>
<td>24.4</td>
<td>7.3</td>
<td>30.5</td>
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</tr>
<tr>
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<tr>
<td>50.00</td>
<td>6.0</td>
<td>7.4</td>
<td>12.6</td>
<td>7.5</td>
</tr>
</tbody>
</table>

* This solution is made up of MnCl₂, KCl, NaCl, CaCl₂ and FeCl₃ such that an equal amount of chloride ion is supplied by each salt. This solution has the same Cl concentration per c.c. as the manganese solution used.
stimulation with low concentrations of manganese chloride and the CaCO₃ markedly reduced of the manganese the toxicity at the higher concentrations. The chloride solution showed less stimulation at lower concentrations but was much less toxic at higher concentrations.

DISCUSSION AND SUMMARY

Studies on some effects of MnSO₄, MnCl₂ and other sulfates and chlorides on the nitrification of dried blood and ammonium sulfate in Carrington loam and Webster loam are reported. Comparisons are made between the soils treated with the manganese salts and the same soils treated with sulfates or chlorides in amounts sufficient to give these soils the same negative ion concentration as the soils treated with manganese. The sulfate solution used was prepared by using MgSO₄, K₂SO₄, Na₂SO₄ and Fe₂(SO)₃, in amounts such that each salt supplied an equal proportion of the sulfate ions per c.c.

The chloride solution used was prepared by using FeCl₃, KCl, NaCl, MgCl₂, CaCl₂, in such amounts that each salt supplied an equal proportion of the chloride ions per c.c.

The results reported show that manganese salts may stimulate or depress nitrification but the degree of
stimulation or depression does not follow regularly with the increase in amount of manganese salts applied. There was no particular concentration of manganese which proved stimulating in all cases nor were the largest amounts applied always the most toxic. However, the toxic effects were more regular than the stimulating effects, usually increasing with the larger applications.

The effects of the sulfate and chloride solutions were similar to those brought about by the manganese sulfate and chloride differing chiefly in degree. The solutions were in all cases less toxic in the large applications than were the manganese salts in the same concentrations. However, there was no regular stimulation nor toxic effect with the small applications.

Lime has a definite influence in reducing the toxic effect of manganese nitrification. This is undoubtedly due to the change in reaction for it is generally true that a basic soil has a higher nitrifying power than a similar acid soil. The sand and solution tests were not extensive enough to warrant general conclusions, however, it is clear that manganese treatments bring about little or no stimulation in nitrification, at least as far as tested in these experiments.
CONCLUSIONS

1. Manganese compounds in small concentrations may stimulate the nitrification of dried blood and ammonium sulfate in soil.

2. There is no regularity of relationship between the concentration of manganese and the stimulation in nitrification.

3. The stimulation in nitrification noted in the presence of manganese salts may be due to the negative ion and not to the manganese.

4. High concentrations of manganese salts retard nitrification but do not stop the process.

5. Lime is very effective in reducing the toxic effects of manganese on nitrification in soil.
ACKNOWLEDGMENT

The author wishes to express his appreciation for suggestions and encouragement given by Dr. P. E. Brown during the progress of this work. He is also indebted to him for reading and correcting the manuscript.
BIBLIOGRAPHY


II. THE ISOLATION OF SOME NITRIFYING ORGANISMS

INTRODUCTION

Much general information regarding the process of nitrification has been available for many years. In fact, the production of nitre by allowing animal and vegetable matter rich in nitrogen to undergo the process of decay under carefully controlled conditions, has been an important industry for ages in India; and even in some of the European countries, especially in France, during the years when she was warring with England whose superior naval power prevented the importation of nitre. Purely empirical instructions were frequently given by the governments in regard to the aeration, temperature, and moisture conditions which should be maintained in the production of nitrates. The nature of the process, however, was unknown.

The early theories of nitrification were chiefly chemical such as those of Kuhlmann (30) and Dumas (15). The oxidation occurring in soil was thought to be similar to catalytic reactions known at the time, the soil acting as a catalyst.

Pasteur (43) suggested the biological theory
in 1862. It slowly gained ground but it was not until the classical experiments of Schloesing and Muntz (46,47) in 1877-78 that this theory was definitely proved. These authors also tried to isolate the specific organisms causing the oxidation but failed as did several other workers of that time.

That nitrification is an important phenomenon in nature is readily seen from the facts, (1) that higher plants with few exceptions grow better with nitrates as a source of nitrogen than with any other source and (2) that nitrates are the end products of protein decomposition in sewage as well as in soil.

REVIEW OF LITERATURE

Interest in the isolation of the nitrifying organisms began with the proof that nitrification is a biological process. Schloesing and Muntz (46,47) succeeded in showing that heat and antiseptics would stop the process and that it would begin again as soon as a very small amount of fresh soil was introduced into the medium. They failed, however, to isolate the organisms responsible for the nitrate production. They believed that the organisms were yeast-like forms. The proved
that they were absent from air, present in small numbers in ordinary water, more abundant in sewage, and very abundant in soil. Ninety degrees centigrade was found sufficient to inhibit the reaction and 100 degrees centigrade invariably killed the organisms. Soils treated with chloroform were slower to regain nitrifying activity than similar soils after being heated.

Heraeus (23) in studying sewage isolated twelve organisms which he called alpha, beta, gamma, etc., of these he claimed the rho, sigma, phi and chi forms had nitrifying powers. His work has not been repeated, and subsequent workers have doubted the validity of his claims.

Warington (55, 56) carried out extensive and careful experiments with nitrifying organisms. He found that nitrification takes place in two stages; first, the oxidation of ammonia to nitrites; and second, the oxidation of nitrites to nitrates. He found further that nitrification would take place in the presence of urea, asparagine, milk and urine.

He isolated some organisms by means of gelatin and agar plates from his nitrifying cultures but they would not nitrify when introduced into mineral solutions. Of the organisms in his cultures some were much like those described later by Winogradsky, but since they were not
isolated by a plate method, they were probably not pure.

The Franklands (16) after two years of study, using chiefly the dilution method, believed they had a pure culture of nitrifiers. They described the organism as a short rod, 0.8 micron long and nearly as thick. They cultivated this organism in mineral solution for nearly three years.

The first American investigators to study the problem were Jordan and Richards (25) who proved that nitrifiers were invariably present in ordinary waters; and although they failed to isolate the organism by the gelatin plate method, they claim to have succeeded by means of dilution. Their criteria of purity were (1) oxidation of ammonia and (2) the microscopic appearance of the organism.

Leone (31) believed that certain organisms may successively produce nitrates and reduce them, according to the environmental conditions.

Winogradsky (60,61) began his studies on the nitrifying organisms in 1890. He had made outstanding contributions to our knowledge of the sulfur and iron bacteria and was attracted to this field perhaps because here was a group of organisms which, like those he had been studying, had the power of oxidizing inorganic
materials. He succeeded in isolating a motile, ovoid, organism which he named *Nitrosomonas* which would oxidize ammonia to nitrites, very rapidly in suitable media and would not grow on the gelatin plate. Five species of organisms which he called alpha, beta, gamma, delta and epsilon, were difficult to eliminate from his enrichment cultures. Of these the beta and epsilon forms were described as follows: "Beta un petit oidium interessant, etrange, n'appartenant probablement pas aux bacteries, mais au groupe des especes bourgeonnantes (Sprosspilze)." The others were a micrococcus, a long rod and a short rod respectively.

By using recrystallized salts and twice distilled water he finally succeeded in eliminating all these forms except the epsilon. To eliminate this organism he seeded some magnesium carbonate sediment from a nitrifying culture on gelatin plates. The epsilon organism grew well forming visible colonies. Here and there on the plates were found carbonate particles remote from the epsilon colonies which by examination were found to be covered with small ovoid organisms. When this organism was transferred to a mineral solution it oxidized ammonia to nitrites and was found to be free from the
epsilon form by its failure to show growth on gelatin. This process has since been known as the inverse gelatin method for isolating nitrifiers.

Winogradsky expressed disappointment that his alpha, beta, etc., forms could not nitrify ammonia. He later isolated other species of the genus *Nitrosomonas*, and also a nitrite-oxidizing organism which he named *Nitrobacter*. This too, was a small ovoid organism whose length was never more than one-half micromillimeter and the thickness much less.

Working alone and with Omeliansky (58,59) he carried out many physiological studies on these organisms. He determined the ratio of nitrogen oxidized to carbon reduced and found it to be 35:1 for *Nitrosomonas* and 40:1 for *Nitrobacter*. By determining the total carbon at the time of inoculation period, he was able to show that the carbon came into the solution in an amount equal to the organic carbon formed. Soluble organic matter was found to be very toxic to these organisms.

Later Winogradsky improved his methods for the isolation of these organisms and used silica gel plates. A finely drawn glass tube or rod was used for transferring the minute microscopic colonies. Inoculations were made
into alkaline broth and if no growth occurred, the cultures were considered pure. It is a significant fact, as pointed out by Gibbs (19), that Winogradsky's work has never been repeated in its entirety!

Godlewsky (20, 21) succeeded in confirming Winogradsky's results with respect to the source of carbon. He also showed that carbon dioxide is necessary for the metabolism of the organisms.

Burri and Stutzer (10, 11) isolated a nitrate-forming organism. They claimed it would grow in broth. However, Winogradsky obtained one of their "pure" cultures and isolated from it not only the nitrate organism but also two other forms. The rather severe criticism by the Russian bacteriologist was answered by Stutzer and Hartleb (50) who pointed out the difficulties which Winogradsky had encountered in his isolation studies. Later Stutzer (49) isolated and studied the nitrifiers and showed that while most organic matter is toxic, soil extract, "abkockung" is not. Still later these authors isolated an organism which they called Nitromicrobium and another called Hypomicrobium. Their description of the latter, as Gowda (22) points out, resembles that of certain actinomyces.

Omeliansky (38) reports some experiments which were designed to show that pure cultures of nitrifiers
could not nitrify urine, urea, egg-albumin, asparagin, or bouillon as had been reported. He took special precautions to eliminate all traces of ammonia from all cultures at the beginning. The organisms isolated by Winogradsky's method were used. No trace of nitrites or nitrates appeared. This worker later, 1899, (39) introduced the gypsum block as a suitable solid substratum for growing these organisms, when the usual solution of salts were used. He found that small droplet-like colonies appeared in 3 to 4 days, and in 10 to 14 days the colonies were 0.25 to 0.50 mm in diameter. He (40) also grew the nitrite-forming organism on pads of high grade filter paper, stitched together with fine thread, sterilized in petri dishes, and soaked with a mineral medium.

Fremlin (18) used agar, gelatin and silica gel plates for isolating the Nitroso-bacterium. His experiments tend to show that the organism often loses its power of oxidizing ammonia when grown in organic media but that this power is regained by passing through sterile soil. Some cultures, however, showed oxidation on the first transfer. Fifty-three culture-flasks were seeded with pieces of beef-broth agar containing the organisms and in 20 of them an oxidation of the nitrogen
occurred. In nineteen similar flasks inoculated with pieces of agar from the same plates but taken from portions of the plates where no growth could be detected, there was no oxidation. The organism studied was ovoid, resembling Winogradsky's Nitrosomonas.

Boullinger and Massol (7,8) using the silica gel plates claim to have isolated both nitrifying organisms. They tested several ammonium compounds in various concentrations and found that 30 grams of ammonium sulfate per liter greatly retarded nitrite formation, and 20 grams of sodium nitrite per liter hindered nitrate formation. Thirty-seven degrees centigrade was found to be the optimum temperature for nitrite formation and at 45 degrees the reaction stopped. For the nitrate former 55 degrees was required to stop the reaction.

These workers tested several materials such as cinders, crushed porcelain, pumice, crushed bricks and sand placed in the medium to increase aeration. Of these materials cinders, "scories," was found to be the best for both nitrite and nitrate formation.

Using Winogradsky's inverse gelatin method, described above Wimmer (57) isolated the two nitrifying organisms which he said were similar to those isolated by Winogradsky and should, therefore, belong to the same
genera. These would not grow in bouillon. He studied the effects of soluble organic matter and found that it was toxic in the higher concentrations. Using sand reduced the toxicity of these organic materials considerably.

Perotti (44) isolated a nitrite-forming coccus by means of the silica gel plate. It was 0.6 to 0.8 micron in diameter and appeared slightly ovoid at times. It was motile by means of one flagellum, gram negative, and was usually found in groups of two to six cells.

While studying some hydrogen-oxidizing organisms, Kaserer (27) isolated two species of organisms which he named Bacillus nitrator and Bacillus azotofluorescens respectively. The first of these he found would oxidize ammonia to nitrates without forming nitrites. The second would oxidize ammonia liberating free nitrogen. These studies have never been confirmed.

Thomsen (51) isolated some nitrifying organisms from sea water. They were like those described by Winogradsky and were found chiefly in waters close to the shore. They were seldom found beyond the depth of 100 meters.

Coleman (12) demonstrated that dextrose in low concentrations in soil and sand was actually stimulating
to nitrification. In higher concentrations it became first indifferent and finally toxic. Sucrose, glycerin, and lactose also were stimulating in low concentrations but less so than dextrose. Peptone and urea were toxic in comparatively low concentrations. The optimum moisture was found to be 16 percent. Coleman emphasized the importance of aeration in the process and he found carbon disulfide very toxic to the organisms.

Owen (42) reports a study on the effects of carbonates on nitrification. It is doubtful if he had pure cultures for his photomicrographs do not indicate it and his methods as described are rather inadequate.

Makrinoff (34) studied the effects of organic substances on nitrifying bacteria grown on gypsum blocks, in soil, and in solutions to which soil had been added. He found that a longer time was needed before oxidation took place in solutions to which considerable soil had been added than in pure mineral solutions. Soil extracts were more toxic in solution cultures than when used with the gypsum blocks. One nitrite culture was obtained from Omeliansky and the author isolated others by means of silica gel plates. The organisms studied were similar to those isolated by Winogradsky.
Millard (37) using the dilution method found that 100,000 nitrifiers were present per gram in the soil studied. There were none present in dung.

Beijerinck (3) isolated a nitrate-forming organism from the soils of Delft by means of silica gel and washed agar plates. It resembled Nitrobacter but would grow in organic media. He found, however, that this organism lost its power to oxidize nitrites when so grown. He named the oxidizing form Nitrobacter oligotrophum and the non-oxidizing form Nitrobacter polytrophum. This latter form did not regain its power to oxidize nitrites after being grown in organic media.

A membrane formed on the surface of his crude cultures which he called mother of nitrate, "Nitratmutter." This contained many organisms including several species of the Actinomycetes. Most of these were readily eliminated in the enrichment cultures but two species, Actinobacillus oligocarbophilus and Actinobacillus paulotrophus, of the family Actinomycetales, and a short rod which he named Bacillus nitroxsus, were harder to eliminate.

Joshi (26) described a nitrifying organism which was undoubtedly one of the Actinomycetes. His description of the culture and his photomicrographs are
quite convincing. This organism produced nitrates from ammonia in Omeliansky's solution. Three rod-shaped organisms, which he called intruders, are mentioned but not described.

He studied the effects of dextrose, asparagine, and urea on the organisms. One-tenth of a gram of dextrose in a 50 c.c. solution stimulated nitrite production while 0.2 gram stopped it; 0.1 gram of asparagine stimulated nitrite production, but 0.2 gram retarded it; both 0.1 and 0.2 gram of urea stimulated nitrite production. Carbon dioxide and coal gas stimulated nitrification and calcium carbonate was found superior to magnesium carbonate as a base. The thermal death point was between 70 and 80 degrees centigrade and the optimum for growth was between 25 and 35 degrees centigrade.

Hopkins and Whiting (24) studied the effects of nitrification on the solubility of phosphates and found that the nitrite-forming organism had a much greater solvent effect on these materials than the nitrate-forming organism.

Russel and Bartow (45) report the isolation of the nitrifiers from activated sludge. The nitrite organism produced 0.1 to 0.2 parts per million of nitrates in sterilized sludge and the two growing together formed
traces of nitrites and nitrates. The fresh sludge in
the same time produced 12 parts per million. The nitrogen
analysis of the sludge used is not given so the results
are difficult to interpret properly.

Allen and Bonazzi (1) conducted experiments on
nitrification. They pointed out the need for physiological
studies on the nitrifying organisms which Bonazzi (4,5,6)
later carried out. This worker isolated a coccus which
formed nitrites quite rapidly and named it *Nitrosococcus*.
He found that aeration, agitation of the cultures, and
free carbon dioxide were necessary for the most rapid
nitrification in liquid media.

The broth test using a loopful of culture for
inoculum, and the microscopic examination of the cultures
were his criteria of purity. This was criticized by
Gibbs (19) who claimed that a larger inoculum than
Bonazzi used namely 1/2 c.c. was necessary to insure the
absence of contaminants.

Gibbs (19) reports the isolation of the
nitrifiers and certain physiological studies. He found
the method used by Bonazzi gave conflicting results but
if it was modified i.e. using one-half c.c. of the culture
as inoculum in the broth test, he obtained consistent
results. However, it is noteworthy that his cultures meeting this condition of purity, rapidly lost their power to oxidize ammonia. His explanation of this fact (physiological degeneration from growing on a solid medium), seems to the present writer wholly inadequate. He describes briefly three forms which were eliminated with some difficulty from his cultures.

Fred and Davenport (17) studied the effects of organic matter on the nitrate former which they isolated by means of dilutions and plating on washed agar. Nährstoff-Heyden solution was inoculated with one-half c.c. of culture and if it remained sterile after two weeks incubation, the culture was considered pure. These investigators say that the nitrate-forming organism will remain alive for two to six weeks and perhaps longer in 1 percent Nährstoff-Heyden, gelatin, peptone, casein, yeast water, milk or distilled water; but they found that the organism dies very rapidly in 1 percent beef extract.

Gowda (22) was unable to obtain consistent results with the broth test. The cultures which were sterile at first were either found to be contaminated later or they would not nitrify. Five organisms, two bacteria and three Actinomyces which were more or less constant contaminants are described. Some physiological
studies are also reported.

Loew (32,33) in 1891 discussed the chemistry of the nitrifying process. He concluded that the following equations probably represent the transformations of nitrite formation and carbon reduction:

1. $2 \text{NH}_3 + 2\text{O}_2 = 2\text{NO}_2 + 4\text{H}$
2. $\text{CO}_2 + 4\text{H} = \text{H}_2\text{O} + \text{HCHO}$

The formaldehyde could be used directly or polymerized to one of the higher carbohydrates, and then be assimilated.

Meyerhof (36) in a series of three papers reports his studies on the respiration of nitrifying bacteria. His organisms were obtained from Omoliansky.

Those interested in the physiology of these organisms will find these papers very interesting. This phase of the subject is also well summarized by Kostytschew (28) and Buchanan and Fulmer (9).
PRELIMINARY TESTS

The object of these tests was to isolate in pure culture some of the nitrifying organisms.

I.

A slightly modified Omeliansky solution was prepared having the following composition: $(\text{NH}_4)_2\text{SO}_4$, 1 gram; $\text{K}_2\text{HPO}_4$, 1 gram; NaCl, 2 grams; MgSO$_4$, 0.5 gram; Fe$_2$(SO$_4$)$_3$, trace; MnSO$_4$, trace; H$_2$O, 1 liter; MgCO$_3$, excess. This is Medium 1. The MgCO$_3$ was sterilized separately as a suspension and added with a sterile pipette. 500 c.c. Erlenmeyer flasks were used in all of these experiments with 100 c.c. of solution.

A few grams of Carrington loam were used as the inoculum and the culture incubated for two weeks. At this time a strong nitrite reaction seemed to indicate that there were enough nitrifiers present to isolate.

Fifteen grams of washed agar were added to a liter of Medium 1, in which 0.5 gram K$_2$CO$_3$ was substituted for the MgCO$_3$. Plates were poured in the usual way. A few strands of sterile glass wool were dipped in the culture and placed on the surface of the plates to see if colonies would form and make it easier to locate them.
In 5 to 7 days colonies appeared all along the glass hairs. They were chiefly yellowish-brown, round or oval, some flat and others slightly raised in the center.

Several of these were picked out with a micro-pipette and inoculated into Medium 1. After shaking, one-half c.c. was inoculated into nutrient broth of the following composition: Beef-extract, 3 grams; peptone, 7 grams; K$_2$CO$_3$, 0.5 gram; H$_2$O, 1 liter; pH, 6.8. Distilled water only was used in these experiments.

All cultures showed oxidation and all showed growth in broth.

II.

It was evident from Test I that there were enough organisms present in the cultures to isolate but the colonies were too numerous and close together to allow isolation of single species. In view of this fact, plates were prepared in the following manner:

Solutions A, B, and C, modifications of those used by Gibbs, (19) were prepared. They had the following composition:
A. K$_2$HPO$_4$, 1.5 gram per 100 c.c.
B. (NH$_4$)$_2$SO$_4$, 1.5 gram per 100 c.c.
  MgSO$_4$, 0.75 gram
  Fe$_2$(SO$_4$)$_3$, trace
  MnSO$_4$, trace
C. NaCl, 3.0 grams per 100 c.c.
  Na$_2$CO$_3$, 1.5 gram per 100 c.c.

These solutions were sterilized separately and added to the plates when pouring the agar, 1 c.c. of each solution per plate. The agar used was one and one-half percent washed agar in distilled water, about 10 c.c. being used per plate. The inoculum 1 c.c. of a dilution of one of the cultures was mixed with the agar by rotating the plates before the agar was allowed to set. They were incubated under a bell jar to check evaporation, and examined from time to time with the microscope.

Microscopic colonies appeared in 10 days. Two kinds were chiefly in evidence. These were (1) spreading, slightly yellow without any definite form, always on the surface; (2) subsurface, oblong, clearly granular, yellowish-brown. The surface colonies were easily transferred with the micropipette but the subsurface colonies were difficult to remove. However, a number of
each type were transferred to Medium 1 and tests made in broth as described in Test 1. All flasks showed oxidation and all broth tubes became cloudy.

Two species of actinomyces were removed from one of these plates and cultivated on nutrient agar, a medium made by adding 15 grams of agar to one liter of nutrient broth. These cultures were replated and kept in the refrigerator until later. They will be called Actinomyces 200 and Actinomyces 300 respectively.

III.

Having failed to obtain pure cultures with washed agar it was decided to try isolation with silica gel plates. These were prepared by the method outlined by Waksman and Carey (53), being dialized, however, with distilled water until free from chlorides and allowed to dry two or three days before adding the nutrient salts. These salts were supplied by adding 1 c.c. of each of solutions A, B and C of Test II to each plate. After allowing the plates to dry again under a cover to keep out the dust, they were carefully flamed and covered with sterile covers. Inoculations were made with sterile pipettes, one-half c.c. of a suspension of organisms being added to the center of the plate and allowing it
Colonies developed somewhat slower on these plates than on washed agar but they were about the same in form and size. In all the first transfers of these colonies oxidation occurred and growth took place in broth.

One plate after nearly one month's incubation showed several quite uniform colonies. They were ovoid, raised in the center, granular as seen with the high power lens, and rather dark brown. These were easily transferred with a micropipette and twelve flasks of Medium 1 were inoculated. All of these cultures showed growth in broth in one week. The oxidation of ammonium sulfate was somewhat slower than in previous tests. At the end of 10 days it was thought well to test these cultures again with nutrient broth. Accordingly, one-half c.c. from each flask was transferred to a broth tube. Six tubes showed rapid growth but the other four remained perfectly clear. After several weeks these cultures were again tested and this time rapid growth occurred in the broth. By this test then these cultures were at first impure then pure and finally impure again. Nutrient agar plates were poured and inoculated from a dilution of culture number 8, one of the four cultures.
just mentioned. Only one type of organism developed. The surface colonies were round, white by reflected light and light blue by transmitted light. The subsurface colonies were ovoid, yellowish-brown, and granular as seen with the high power dry lens. The organism was a coccus and is called coccus 800 in this paper.

EXPERIMENTAL

Before presenting the results of this experiment a short discussion of the problem will be given.

From the brief review of the subject given earlier certain facts stand out rather prominently. First, the so-called contaminators have been noted by all students of the subject. These organisms have persisted in dilution tests almost indefinitely, in fact the Franklands (16) are the only investigators who believe they obtained pure cultures by this method and they carried on their studies for two years. Winogradsky found it impossible to eliminate the last of these organisms without plating. Why do these organisms persist in mineral solutions?

Second, the broth test has not given consistent results. If Gibbs (19) contention is correct that at
least one-half c.c. of the culture must be transferred to make the test reliable, then all previous work must be unreliable, for no one used such large inoculations. Gowda (22) could not obtain consistent results with this test even when he used one-half c.c. and it is no less significant that the cultures of Gibbs which met his criterion of purity, in most cases lost their power to oxidize ammonia as well. In Test II just discussed, the cultures were first impure, later pure and finally impure again by this test.

Third, by what energy do the contaminating forms continue to function in a mineral medium? One would infer that they multiply or it should be a rather simple matter to eliminate them from a culture by the dilution method. There is only one plentiful supply of energy in these solutions, namely, ammonium compounds. It is hardly likely that the true nitrifiers could reduce enough carbon, resynthesize it, and make it available to keep the contaminating organisms not only alive but multiplying so rapidly that they cannot be eliminated by dilution methods.

The last paragraph, however, contains an assumption, for no one as far as known has proved that

* See also Waksman (54) page 69.
contaminating forms actually multiply in mineral solutions. To answer this question, the following tests were made.

**Experiment 1.**

(a). A flask of Medium 1 was inoculated with 1 c.c. of a culture which showed rapid oxidation of ammonia and was impure by the broth test. Counts were made by means of nutrient agar plates at weekly intervals. These are the results:

<table>
<thead>
<tr>
<th>Age when counted</th>
<th>Number per c.c.</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>66,600</td>
</tr>
<tr>
<td>1 week</td>
<td>1,366,800</td>
</tr>
<tr>
<td>2 weeks</td>
<td>553,000</td>
</tr>
<tr>
<td>3 weeks</td>
<td>155,000</td>
</tr>
<tr>
<td>less than 8 weeks</td>
<td>10,000</td>
</tr>
</tbody>
</table>

These results show that there was a rapid increase in numbers the first week followed by an irregular falling off. The data are too meagre to permit of any general conclusions, but there is an interesting indication shown.

(b). Would a contaminating organism growing on nutrient agar multiply when placed in a mineral solution? Coccus 800 was chosen in the attempt to
answer this question.

A flask of Medium 1 was prepared in the usual way and inoculated with a needle from an agar slant. Counts were made at the time of inoculation and at weekly intervals. The following are the results:

<table>
<thead>
<tr>
<th>Age when counted</th>
<th>Numbers per c.c.</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>135,300</td>
</tr>
<tr>
<td>Less than 1 week</td>
<td>1,000</td>
</tr>
<tr>
<td>Less than 2 weeks</td>
<td>100</td>
</tr>
<tr>
<td>9 weeks</td>
<td>145,000</td>
</tr>
</tbody>
</table>

The organisms died off rapidly in this culture more than 99.24 percent dying off the first week and more than 99.92 percent had died by the end of two weeks. Counts were not made again until the end of 8 weeks at which time an increase in numbers had occurred. Evidently there was an adaptation taking place in this solution. At the end of 11 weeks nitrites appeared in the solution.

The inoculum was very large in this case. If a small inoculum had been used the results might have been different. It is possible that one-half c.c. could be withdrawn for the broth test and no organisms be transferred. Such a condition would account for the results noted in Experiment 3.
(c). Would it be possible to prevent the rapid dying off noted in the last experiment? It was thought probable that an organic carbon source would be more easily utilized and reduce the high death rate.

A flask of Medium 1 was prepared in the usual way and made up to 0.25 percent dextrose. Two colonies from a plate used in counting in Experiment 1, (a) were mixed with the needle and a small portion transferred to the flask. Counts were then made to determine the effects on multiplication. The results follow:

<table>
<thead>
<tr>
<th>Age when counted</th>
<th>Organisms per c.c.</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>316,000</td>
</tr>
<tr>
<td>1 week</td>
<td>4,300,000</td>
</tr>
<tr>
<td>2 weeks</td>
<td>54,650,000</td>
</tr>
<tr>
<td>3 weeks</td>
<td>167,665,000</td>
</tr>
</tbody>
</table>

The last plates were counted when the culture was 4 weeks old. The culture gave a very strong test for nitrites at this time and when analysed a few weeks later contained 7.407 p.p.m. of nitrite nitrogen. These organisms had developed on a plate of nutrient agar, the medium recommended for tests of purity; for one week and when transferred to Medium 1 with 0.25 percent dextrose, had produced oxidation. This was not a pure culture but

* See Waksman (54) page 69.
fact that it grew on nutrient agar, developed colonies, and later produced oxidation showed that soluble organic matter was not toxic to these organisms.

Experiment 2.

The last experiment seemed to indicate that the organisms growing in broth might be able to oxidize ammonia if they were grown under suitable conditions. It was thought worth while to test a number of broth cultures from various silica gel and washed agar plates. The cultures need a brief description in order to make the next table clear.

1. A loopful from the culture described in experiment (c) was introduced with Medium 1 + 0.25 percent dextrose.

2. Mixed colonies from a plate in experiment (c) were used in the attempt to repeat that experiment.

3. The same as 2, except that a single colony was used in this case.

4. The same inoculum as 1 on Medium 1 without dextrose.

5. Coccus 800 described in Test III, introduced into Medium 1 + .25 percent dextrose.

6. The broth tube from one of the cultures
described in Test III showed a peculiar growth. Small butter-like globules formed around the surface of the tube. A similar growth was noticed by Govda (22). One of these was used for the inoculum in 6. 6a contained 0.25 percent dextrose while 6b contained no organic matter.

7. A broth culture kept from the first isolations described in Test I was used. 7a contained dextrose, 7b contained no organic matter.

8. Actinomyces 300 mentioned in Test II was used for inoculum. 8a contained 0.25 percent dextrose, 8b contained no organic matter.

9. An actinomyces developed on one of the plates in experiment (c). This was used for inoculum and will be called Actinomyces 400 in all the tables. Both solutions were Medium 1 with 0.25 percent dextrose added.

10. This was Actinomyces 200 mentioned in Test II. Both flasks contained Medium 1 with 0.25 percent dextrose added.

The checks showed a trace of nitrite by the sulphanilic acid and alpha naphthyamine reaction but it was far less intense than the reaction shown by the cultures.

Numbers la and lb indicate that the culture had not lost its power to nitrify but it grew very slowly.


<table>
<thead>
<tr>
<th>Number</th>
<th>Days before nitrites</th>
<th>Number</th>
<th>Days before nitrites</th>
</tr>
</thead>
<tbody>
<tr>
<td>1a, 1b</td>
<td>5, 5</td>
<td>7a, 7b</td>
<td>81, 81</td>
</tr>
<tr>
<td>2a, 2b</td>
<td>81, 81</td>
<td>8a, 8b</td>
<td>81, 81</td>
</tr>
<tr>
<td>3a, 3b</td>
<td>81, 81</td>
<td>9a, 9b</td>
<td>18, 81</td>
</tr>
<tr>
<td>4a, 4b</td>
<td>81, 81</td>
<td>10a, 10b</td>
<td>81, 81</td>
</tr>
<tr>
<td>5a, 5b</td>
<td>5, 81</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6a, 6b</td>
<td>22, 81</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
in a purely mineral medium as shown by Numbers 4a and 4b. Numbers 2a and 2b did not give the same results as in Experiment 1 (c). There was an oxidation, however, but it required a longer time. Cultures 3 and 4 showed the same reaction as Number 2.

In the case of 5a and 5b no explanation can be offered. These cultures were apparently as nearly alike as any at the beginning and subsequent subcultures did not reveal any foreign organisms or differences in growth characteristics.

6a and 6b clearly show the effects of the sugar but this was not the case with 7 and 8 where no differences were noted.

Number 10 showed a positive reaction for nitrates to diphenalamine after 25 days but since it did not seem to increase in intensity it was not considered significant. Only tests which were very definite were recorded, for small amounts of nitrites may be absorbed by these solutions as shown by later experiments.

Experiment 3.

Upon examining some silica gel plates which had been kept under bell jars for three months, some white colonies of Actinomyces were noticed. (Plates I and II) The colonies closely resembled each other so it
was thought probable that they were the same organism. A few threads were carefully removed from the surface of one of the colonies to nutrient agar. After growth occurred the organism was replated to insure a pure culture and was then used in the following experiment. It is called Actinomyces 600 in this paper.

Six flasks of Medium 1 were prepared in the usual way. These were inoculated with a needle from an agar slant, incubated at room temperature for 38 days and analysed for nitrites.

Table II gives the results.

This organism grew well on nutrient agar and when transferred to Medium 1 with or without dextrose, it was able to utilize ammonium sulfate as a source of energy. The carbohydrate evidently stimulated growth as an examination of the flasks showed the growth to be roughly proportional to the dextrose content. This was not the case with oxidation, however, for there was little difference between cultures 2 and 3.

These cultures were tested for nitrates as follows: 10 c.c. of the culture was withdrawn, treated with a few crystals of urea and 5 c.c. of dil. H₂SO₄. When the reaction was complete the absence of nitrite was demonstrated by the sulphinilic acid and alpha
### TABLE II

The Effects of Dextrose on the Oxidation of Ammonia by Actinomyces 600.

<table>
<thead>
<tr>
<th>Number</th>
<th>Percent</th>
<th>Nitrite N.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Check</td>
<td>0.00</td>
<td>trace</td>
</tr>
<tr>
<td>Check</td>
<td>0.00</td>
<td>trace</td>
</tr>
<tr>
<td>1a</td>
<td>0.00</td>
<td>3.1</td>
</tr>
<tr>
<td>1b</td>
<td>0.00</td>
<td>4.4</td>
</tr>
<tr>
<td>2a</td>
<td>0.10</td>
<td>20.9</td>
</tr>
<tr>
<td>2b</td>
<td>0.10</td>
<td>16.6</td>
</tr>
<tr>
<td>3a</td>
<td>0.25</td>
<td>10.4</td>
</tr>
<tr>
<td>3b</td>
<td>0.25</td>
<td>34.7</td>
</tr>
</tbody>
</table>
naphthylamine test. All the cultures showed the presence of nitrates with the diphenylamine test. Can the organism utilize nitrite nitrogen? It was decided to test this culture to learn if this was the case.

Experiment 4.

A medium was prepared having the following composition:

\[
\begin{align*}
\text{NaNO}_2 & \quad - \quad - \quad - \quad - \quad - \quad 1.0 \text{ gram} \\
\text{Na}_2\text{CO}_3 & \quad - \quad - \quad - \quad - \quad - \quad 1.0 \text{ gram} \\
\text{K}_2\text{HPO}_4 & \quad - \quad - \quad - \quad - \quad - \quad 0.5 \text{ gram} \\
\text{NaCl} & \quad - \quad - \quad - \quad - \quad - \quad 0.5 \text{ gram} \\
\text{MgSO}_4 & \quad - \quad - \quad - \quad - \quad - \quad 0.3 \text{ gram} \\
\text{Fe}_2(\text{SO}_4)_3 & \quad - \quad - \quad - \quad - \quad - \quad \text{trace} \\
\text{MnSO}_4 & \quad - \quad - \quad - \quad - \quad - \quad \text{trace} \\
\text{H}_2\text{O} & \quad - \quad - \quad - \quad - \quad - \quad 1000 \text{ c.c.}
\end{align*}
\]

This is called Medium 2 in this paper. 100 c.c. portions were placed in 500 c.c. Erlenmeyer flasks and sterilized. The dextrose treatments were added with a sterile pipette after cooling. After 38 days the cultures were analyzed for nitrates. The following method was used.

The culture was treated with a few crystals of urea and then 25 c.c. dil. H\(_2\)SO\(_4\). The absence of nitrite
was shown by the sulphanilic acid alpha naphthylamine test. A small portion of silver sulfate was then added to precipitate the chlorides and sufficient calcium hydroxid to neutralize the acidity. The cultures were then filtered and an aliquot taken and analyzed by the phenoldisulphonic acid method. The results are given in table III.

The checks contained only a trace of nitrate nitrogen which was somewhat unusual as seen by some of the later experiments.

The growth was about the same as those with ammonium sulfate and roughly in proportion to the dextrose content.

The case of la and lb is interesting. The growth was plainly visible on the surface of the solution. There was very little organic matter present in the medium for only the best chemicals were used and the water was distilled from a dichromate solution. While the oxidation was slow it was none the less definite.

**Experiment 5.**

In Experiment 2 a culture was mentioned which showed butterlike globules around the surface when grown in broth. One of these globules was removed to a
<table>
<thead>
<tr>
<th>Number</th>
<th>Dextrose</th>
<th>Nitrates</th>
</tr>
</thead>
<tbody>
<tr>
<td>Check</td>
<td>none</td>
<td>trace</td>
</tr>
<tr>
<td>Check</td>
<td>none</td>
<td>trace</td>
</tr>
<tr>
<td>1a</td>
<td>none</td>
<td>1.66</td>
</tr>
<tr>
<td>1b</td>
<td>none</td>
<td>1.25</td>
</tr>
<tr>
<td>2a</td>
<td>0.10</td>
<td>1.20</td>
</tr>
<tr>
<td>2b</td>
<td>0.10</td>
<td>1.71</td>
</tr>
<tr>
<td>3a</td>
<td>.25</td>
<td>1.20</td>
</tr>
<tr>
<td>3b</td>
<td>.25</td>
<td>1.53</td>
</tr>
</tbody>
</table>
dilution flask, shaken and some plates made with nutrient agar. Only one type colony appeared. These were usually round, gray, shallow. The organism was ovoid and was as a rule in chains of 2 to 6 cells. There was another organism in the broth tube but I did not succeed in isolating it at this time. The ovoid organism was re-plated to insure a pure culture and used in the following tests. It will be called Bacterium 500 in this paper.

In Experiment 2 an organism called Actinomyces 300 was mentioned. It too was used in these tests.

Flasks of Medium 1 and Medium 2 were prepared in the usual way and made up to 0.25 percent dextrose, sterile checks being made at the same time. Table IV shows the results after 62 days.

No growth occurred in any of the nitrite flasks so it was clear that these organisms could not use this source of nitrogen, at least under the conditions of this experiment.

**Experiment 6**

In Experiment 2 Number 9 was an Actinomyces called 400 in this paper. When this organism is grown in a medium containing dextrose it excretes a pigment which appears pink at first but turns brown if sufficient
dextrose is present. The depth of color seems to be in proportion to the amount of dextrose present. It was found in the first tests in Experiment 5 that the duplicates did not agree. This experiment was outlined to try to find out if this organism would oxidize nitrogen in sufficient amounts to be determined quantitatively. Number 1 was inoculated with a mass of growth from the surface of culture 9 Experiment 5, Number 2 was inoculated from a tube of Dextrose agar. This medium was prepared by substituting 0.5 gram K$_2$CO$_3$ for MgCO$_3$ in Medium 1, adding ten grams of dextrose, 15 grams washed agar and sterilizing in the autoclave 15 minutes at 15 pounds. Number 3 was an extra flask which contained 0.25 percent dextrose and was inoculated as Number 1 and placed with the cultures. Table V gives the results after 71 days.

This table shows that the source of the inoculum had no effect and that the higher dextrose content gave much the highest oxidation. It should be mentioned that these cultures were clarified with G Elf black before the nitrates were determined, as the pink pigment seriously interferes with the determination. They are easily clarified by shaking with a small portion of G Elf black and then filtering.
TABLE V

Effects of Dextrose on Oxidation of Ammonium Sulfate by Actinomycetes 400.

<table>
<thead>
<tr>
<th>Number</th>
<th>Dextrose</th>
<th>Nitrite N.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Check</td>
<td>0.0</td>
<td>trace</td>
</tr>
<tr>
<td>Check</td>
<td>0.0</td>
<td>trace</td>
</tr>
<tr>
<td>1</td>
<td>.01</td>
<td>5.55</td>
</tr>
<tr>
<td>1</td>
<td>.01</td>
<td>4.35</td>
</tr>
<tr>
<td>2</td>
<td>.01</td>
<td>5.27</td>
</tr>
<tr>
<td>2</td>
<td>.01</td>
<td>4.55</td>
</tr>
<tr>
<td>3</td>
<td>0.25</td>
<td>40.00</td>
</tr>
</tbody>
</table>
Experiment 7.

This was outlined to learn which of the three organisms so far not tested would grow in Medium 2 when 0.25 percent dextrose is added. Culture flasks were prepared as outlined in Experiment 4. It was found that Actinomyces 400 and Coccus 800 made rapid growth but Actinomyces 200 did not grow at all. Actinomyces 400 produced a pigment similar in every way to that produced in Medium 1 with dextrose added. Coccus 800 produced a slimy mass of growth covering the bottom of the flask. Actinomyces 400 and Coccus 800 both gave positive tests for nitrates but no quantitative determinations were made.

Experiment 8.

The first cultures of 800 in Experiment 2, Number 5, the duplicates did not agree. In one flask there was rapid oxidation while in the other it was slow. It was decided to test several cultures of this organism to learn whether low concentrations of certain organic compounds would stimulate its activity. Several flasks of Medium 1 were prepared in the usual way and sterilized. The various organic constituents were then added from
suitable solutions with a sterile pipette.

In order to have the inoculum equal in all flasks a suspension of the organism was made in a flask of sterile medium and inoculations made with sterile 1 c.c. pipettes. Counts were made to determine the number introduced into each flask. This was found to be 64,500,000 which is a very large inoculum. In Experiment 2 the inoculations were probably not equal and it was thought this might account for the disagreement of duplicates.

Table VI shows the treatments and results after 120 days incubation at room temperature in the dark.

This table shows that very small amounts of peptone stimulated oxidation but dextrose depressed it. Dextrose and peptone together had about the same effect as peptone alone.

The nitrite added as shown in the last part of the table, was equal to 0.01 c.c. of Medium 2 per 100 c.c. of culture. This is equal to 0.002 p.p.m. nitrite nitrogen per c.c., an amount too small to influence the results. Where no dextrose was present the trace of nitrite gave the highest single oxidation but where dextrose was present, the oxidation fell off quite
### TABLE VI

The Effects of Dextrose and Peptone in Low Concentrations on the Nitrification of Ammonium Sulfate by Coccus 800.

<table>
<thead>
<tr>
<th>Flask No.</th>
<th>Dextrose</th>
<th>Peptone</th>
<th>Added</th>
<th>Nitrite N. after 120 days p.p.m.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Check</td>
<td>Percent</td>
<td>Percent</td>
<td></td>
<td>Nitrite N.</td>
</tr>
<tr>
<td>1</td>
<td>none</td>
<td>none</td>
<td>none</td>
<td>trace</td>
</tr>
<tr>
<td>2</td>
<td>.0025</td>
<td>none</td>
<td>none</td>
<td>trace</td>
</tr>
<tr>
<td>3</td>
<td>.0050</td>
<td>none</td>
<td>none</td>
<td>trace</td>
</tr>
<tr>
<td>4</td>
<td>.0100</td>
<td>none</td>
<td>none</td>
<td>trace</td>
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<td>5</td>
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<td>6</td>
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<td>.0100</td>
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<td>none</td>
<td>trace</td>
</tr>
<tr>
<td>11</td>
<td>.0025</td>
<td>.0025</td>
<td>none</td>
<td>trace</td>
</tr>
<tr>
<td>12</td>
<td>.0050</td>
<td>.0050</td>
<td>none</td>
<td>trace</td>
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<td>18</td>
<td>.0200</td>
<td>none</td>
<td>trace</td>
<td>1.32</td>
</tr>
</tbody>
</table>

Note: Dextrose and peptone added to check with nitrite N. after 120 days p.p.m.
regularly with the increase in dextrose. It should be emphasized that aeration was poor and no attempt was made to stimulate the oxidation of large amounts of ammonia. 500 c.c. Erlenmeyer flasks were used and 100 c.c. Erlenmeyer flasks were used and 100 c.c. of medium placed in each. The cultures were incubated in two large drawers.

The amounts oxidized are small but none the less definite. It is evidently true that a basic solution will absorb small amounts of nitrites as pointed out by Jordan and Richards (25). These investigators found that as much as 0.08 p.p.m. of nitrite nitrogen was absorbed by one of their flasks in 90 days. They say that larger amounts are at times absorbed but give no figures. In the work of the writer large amounts of nitrite nitrogen were absorbed in some of the later experiments but in every case checks have been prepared and analysed to insure a correct interpretation of the data secured.

Experiment 9.

A. To test the influence of free carbon dioxid on oxidation of ammonia a number of flasks of Medium 1 were prepared in the usual way. One-hole stoppers
which would fit the flasks were fitted with freshly filled soda lime tubes. The flasks from which it was desired to keep the CO₂ of the air were closed with these stoppers the cotton plugs being left in the lower part of necks of the flasks. Paraffin was used to insure complete sealing of the flasks except of course the opening through the soda lime tubes. It was found necessary to incubate the CO₂ free cultures in the open laboratory since they were too high for the incubator. This might have made a slight difference in temperature. Otherwise the conditions were alike for all cultures. They were incubated 57 days. The results are recorded in table VII.

In most cases the nitrite reaction was stronger in the cultures than in the checks but it was not thought worth while to try to record differences which could not measured quantitatively. In the case of Coccus 800 the carbon dioxid seems to be important in stopping the oxidation. Unfortunately one of these cultures was spoiled by a contamination so only one flask serves for comparison. The mold seemed to stop the growth entirely.

With Actinomyces 200 the absence of carbon dioxid influenced the oxidation slightly but this was
TABLE VII

The Influence of Free CO₂ on the Oxidation of Ammonia and Nitrites by Certain Microorganisms.

<table>
<thead>
<tr>
<th>Organism :</th>
<th>Carbon : Dextrose : 0.10</th>
<th></th>
<th>Nitrite : nitrogen : Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>check</td>
<td>present</td>
<td>absent</td>
<td>trace</td>
</tr>
<tr>
<td>check</td>
<td>present</td>
<td>present</td>
<td></td>
</tr>
</tbody>
</table>

Coccus 800
- present absent
- present absent
- present absent
- present absent
- present absent
- absent present
- absent present
- absent present
- absent present
- absent present
- absent present

A. 200
- present absent
- present absent
- present absent
- present absent
- present absent
- absent present
- absent present
- absent present
- absent present
- absent present

A. 300
- present absent
- present absent
- present absent
- present absent
- present absent
- absent present
- absent present
- absent present
- absent present
- absent present

A. 400
- present absent
- present absent
- present absent
- present absent
- present absent
- absent present
- absent present
- absent present
- absent present
- absent present

Remarks: contaminated, mold
<table>
<thead>
<tr>
<th>Sample</th>
<th>Concentration</th>
<th>Presence</th>
<th>Absence</th>
<th>Test Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>A.</td>
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<td>present</td>
<td>absent</td>
<td>trace</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
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<td>200</td>
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<td>200</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>present</td>
<td>present</td>
<td>5.55</td>
</tr>
<tr>
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<td>200</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>absent</td>
<td></td>
<td>4.84</td>
</tr>
<tr>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
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<td>300</td>
<td>present</td>
<td>absent</td>
<td>trace</td>
</tr>
<tr>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
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<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>300</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>300</td>
<td>present</td>
<td>present</td>
<td>5.00</td>
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<td>300</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>300</td>
<td>absent</td>
<td>present</td>
<td>3.58</td>
</tr>
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<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>400</td>
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<td></td>
<td></td>
</tr>
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<td>400</td>
<td></td>
<td></td>
<td></td>
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<td></td>
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<td>400</td>
<td></td>
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</tr>
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<td></td>
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<td>absent</td>
<td>present</td>
<td>3.58</td>
</tr>
<tr>
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<td></td>
<td></td>
<td></td>
</tr>
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<td>Bact.</td>
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<td>absent</td>
<td>trace</td>
</tr>
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<td></td>
<td>500</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>absent</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>500</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>present</td>
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<td>5.00</td>
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<td>500</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>absent</td>
<td>present</td>
<td>3.58</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A.</td>
<td>600</td>
<td>present</td>
<td>absent</td>
<td></td>
</tr>
<tr>
<td></td>
<td>600</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>600</td>
<td>absent</td>
<td></td>
<td>trace</td>
</tr>
<tr>
<td></td>
<td>600</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>600</td>
<td></td>
<td></td>
<td>contaminated, mold</td>
</tr>
<tr>
<td></td>
<td>600</td>
<td></td>
<td></td>
<td>10.00</td>
</tr>
</tbody>
</table>

Flask broken

Trace
probably due to better aeration in the flasks with free air.

Actinomyces 300 showed good growth in the flasks containing dextrose but organism was only beginning to utilize ammonia as a source of energy.

Actinomyces 400 was favorably affected by dextrose but was not influenced very much by the absence of carbon dioxide.

Bacterium 500 was similar to Actinomyces 300 in all respects except that the growth was not so evident as in the case of the Actinomyces.

Actinomyces 600 was completely checked in the absence of both dextrose and carbon dioxide. Much of this was probably due to aeration. With dextrose the differences were not significant as the duplicates did not agree.

B. Since it was shown previously that only three of these organisms can grow in a nitrite solution, only these organisms were studied. The cultures were prepared in the usual manner using Medium 2 and carbon dioxide excluded with soda lime tubes as described above. The cultures were analyzed as follows: They were first filtered and treated with a few crystals of urea, shaken and 25 c.c. of 1:1 sulfuric acid added. After being
allowed to stand for some time, one c.c. was withdrawn from each flask and tested for the absence of nitrites. The cultures were then neutralized with NaOH and evaporated to about one-half volume. Two or three grams of sodium peroxide were then cautiously added and the flasks slowly heated. When almost dry, they were transferred to distilling flasks with 300 c.c. distilled water, treated with Deverad's alloy and distilled into 1/50 normal acid. The excess acid was then titrated and the calculations made.

Perhaps the outstanding criticism of the data presented in table VIII is the lack of close agreement of the duplicate checks. The error if there is any is probably that it is too high. 600a agrees with the first of the checks and in this culture no growth was visible. With the exception of this culture, all showed growth and the table shows that oxidation occurred in every other case when compared to this check. Even assuming that the highest of the checks was the average for all flasks there was still considerable oxidation produced by these organisms.

Strangely the averages are higher where carbon dioxid was absent in all cases except one, namely Coccus 800 without dextrose. This is extraordinary too
TABLE VIII

The Effects of Carbon Dioxide and Dextrose on the Oxidation of Nitrites by Pure Cultures of Cococcus 800, Actinomyces 400 and Actinomyces 600.

<table>
<thead>
<tr>
<th>Organism</th>
<th>Carbon</th>
<th>Dextrose</th>
<th>Nitrate N. p.p.m.</th>
<th>Average:formed*</th>
</tr>
</thead>
<tbody>
<tr>
<td>check</td>
<td>present</td>
<td>dextrose</td>
<td>7.00</td>
<td></td>
</tr>
<tr>
<td>check</td>
<td></td>
<td></td>
<td>14.00</td>
<td>10.50</td>
</tr>
<tr>
<td>Cococcus 800a</td>
<td>present</td>
<td>absent</td>
<td>15.40</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>19.60</td>
<td>17.50</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>14.00</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>15.40</td>
<td>14.70</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>19.60</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>12.60</td>
<td>16.10</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>22.40</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>16.80</td>
<td>19.60</td>
</tr>
<tr>
<td>A. 400a</td>
<td>present</td>
<td>absent</td>
<td>22.40</td>
<td></td>
</tr>
<tr>
<td>400b</td>
<td></td>
<td></td>
<td>21.00</td>
<td>21.70</td>
</tr>
<tr>
<td>400c</td>
<td>absent</td>
<td></td>
<td>28.00</td>
<td></td>
</tr>
<tr>
<td>400d</td>
<td></td>
<td></td>
<td>19.60</td>
<td>23.80</td>
</tr>
<tr>
<td>400e</td>
<td>present</td>
<td>present</td>
<td>22.40</td>
<td></td>
</tr>
<tr>
<td>400f</td>
<td></td>
<td></td>
<td>16.80</td>
<td>19.60</td>
</tr>
<tr>
<td>400g</td>
<td>absent</td>
<td></td>
<td>spoiled</td>
<td></td>
</tr>
<tr>
<td>400h</td>
<td></td>
<td></td>
<td>32.20</td>
<td>32.20</td>
</tr>
<tr>
<td>600a</td>
<td></td>
<td>absent</td>
<td>7.00</td>
<td>14.00</td>
</tr>
<tr>
<td>600b</td>
<td></td>
<td></td>
<td>21.00</td>
<td></td>
</tr>
<tr>
<td>600c</td>
<td>absent</td>
<td></td>
<td>21.00</td>
<td></td>
</tr>
<tr>
<td>600d</td>
<td></td>
<td></td>
<td>14.00</td>
<td>17.50</td>
</tr>
<tr>
<td>600e</td>
<td>present</td>
<td>present</td>
<td>29.40</td>
<td></td>
</tr>
<tr>
<td>600f</td>
<td></td>
<td></td>
<td>18.20</td>
<td>23.80</td>
</tr>
<tr>
<td>600g</td>
<td>absent</td>
<td></td>
<td>32.20</td>
<td></td>
</tr>
<tr>
<td>600h</td>
<td></td>
<td></td>
<td>25.20</td>
<td>28.70</td>
</tr>
</tbody>
</table>

* This column is obtained by subtracting the average of the checks from the average of the various duplicates.
because the aeration was much poorer in these flasks.

**Experiment 10.**

It was found in Experiment 9, that the absence of organic matter and free CO₂ completely checked the oxidation of ammonia by Actinomyces 600. To test this result more closely, the following experiment was carried out.

Fifty c.c. of Medium 1 was placed in each of 8 250 c.c. Erlenmeyer flasks. Various amounts of dextrose was added and the flasks autoclaved. After inoculation with a suspension of organisms, the cultures were incubated in a large dessicator over strong NaOH. After 46 days the cultures were analyzed for nitrites. The results are recorded in table IX.

The table indicates that there were nitrites in the medium, for there could not have been any absorption from the air. Sample 1 checks the results reported in table VII. While growth was evident in both flasks, it was poor and no oxidation took place. There is little difference in the rate of oxidation in the presence of 0.10 percent and 0.25 percent dextrose. There was a slight difference in the amount of growth, however, more being present with the higher percentage
TABLE IX

The Effects of the Absence of Free CO₂ on the Nitrification of Ammonium Sulfate by Actinomyces 600.

<table>
<thead>
<tr>
<th>Number</th>
<th>dextrose</th>
<th>Nitrite : N. p.p.m.</th>
<th>Average formed: p.p.m.</th>
</tr>
</thead>
<tbody>
<tr>
<td>check</td>
<td>none</td>
<td>0.095</td>
<td></td>
</tr>
<tr>
<td>check</td>
<td>&quot;</td>
<td>0.075</td>
<td>0.085</td>
</tr>
<tr>
<td>1</td>
<td>none</td>
<td>0.044</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>&quot;</td>
<td>0.082</td>
<td>0.063 0.022</td>
</tr>
<tr>
<td>2</td>
<td>0.10</td>
<td>2.625</td>
<td>3.187 3.102</td>
</tr>
<tr>
<td>2</td>
<td>0.10</td>
<td>3.750</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>0.25</td>
<td>4.540</td>
<td>4.200 4.115</td>
</tr>
<tr>
<td>3</td>
<td>0.25</td>
<td>3.861</td>
<td></td>
</tr>
</tbody>
</table>

* This column is obtained by subtracting the average of the checks from the average of the various duplicates.*
of dextrose. The experiment clearly shows that this organism can utilize organic carbon and at the same time oxidize ammonium sulfate.

Experiment II.

A. To learn what concentration of dextrose would stimulate the greatest oxidation with Actinomyces 500, 28 flasks were prepared, 14 with Medium 1 and 14 with Medium 2, in the usual way. Unfortunately the checks for the series with Medium 2 was lost so this series was not analyzed. The cultures were incubated at 30°C. The results with ammonium sulfate after 19 days are given in table X. This experiment slightly modified was later repeated with other organisms previously mentioned.

The amount of growth as shown by the appearance of the flasks increased regularly with the increase of sugar from none to 0.1 percent. There was a decided break at this point, that is the flask having 0.5 percent dextrose had a much greater increase in growth over the one having 0.1 percent than this culture had over the one having 0.01 percent dextrose. The increase in growth from 0.5 percent to 2 percent was quite gradual.
### TABLE X

The Effects of Various Concentrations of Dextrose on the Oxidation of Ammonium Sulfate by Actinomyces 600.

<table>
<thead>
<tr>
<th>Number</th>
<th>Dextrose (p.p.m.)</th>
<th>Nitrite Percent nitrogen</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>check</td>
<td>none</td>
<td>0.00</td>
<td></td>
</tr>
<tr>
<td>check</td>
<td></td>
<td>0.00</td>
<td></td>
</tr>
<tr>
<td>1a</td>
<td>0.000</td>
<td>0.58</td>
<td></td>
</tr>
<tr>
<td>1b</td>
<td>0.000</td>
<td>0.71</td>
<td></td>
</tr>
<tr>
<td>2a</td>
<td>0.001</td>
<td>0.64</td>
<td></td>
</tr>
<tr>
<td>2b</td>
<td>0.001</td>
<td>0.73</td>
<td></td>
</tr>
<tr>
<td>3a</td>
<td>0.01</td>
<td>15.00</td>
<td></td>
</tr>
<tr>
<td>3b</td>
<td>0.01</td>
<td>13.60</td>
<td></td>
</tr>
<tr>
<td>4a</td>
<td>0.1</td>
<td>6.67</td>
<td></td>
</tr>
<tr>
<td>4b</td>
<td>0.1</td>
<td>12.00</td>
<td></td>
</tr>
<tr>
<td>5a</td>
<td>0.5</td>
<td>18.75</td>
<td>contaminated, mold</td>
</tr>
<tr>
<td>5b</td>
<td>0.5</td>
<td>trace</td>
<td></td>
</tr>
<tr>
<td>6a</td>
<td>1.0</td>
<td>trace</td>
<td></td>
</tr>
<tr>
<td>6b</td>
<td>1.0</td>
<td>&quot;</td>
<td></td>
</tr>
<tr>
<td>7a</td>
<td>2.0</td>
<td>&quot;</td>
<td></td>
</tr>
<tr>
<td>7b</td>
<td>2.0</td>
<td>&quot;</td>
<td></td>
</tr>
</tbody>
</table>
Flask 5a was quickly over run with a mold the first few days. Then the mold growth decreased and *Actinomyces* 600 began to grow rapidly. Comparing this with the duplicate it seems that the organism prefers dextrose as a source of energy but can use ammonium sulfate when the sugar becomes limited. The mold evidently used up the dextrose quite rapidly and then the development ceased. *Actinomyces* 600 was able to utilize the only other source of energy in the culture, ammonium sulfate.

With the higher concentrations of dextrose there was a mere trace of nitrite produced. The growth was very much denser, however, than with low concentrations of dextrose forming a heavy mat of growth on the entire surface of the cultures.

The growth on the nitrite medium was similar to that on ammonium sulfate. There seems to be no difference in the utilization of nitrite nitrogen and ammonia nitrogen by this organism.

B. In part A. of this experiment, only a 19 days incubation period was employed. Would this organism oxidize ammonium in the presence of higher concentrations of dextrose if more time were allowed? To answer this question, the following tests were made:
Cultures were prepared in the usual way using Media 1 and 2. The cultures were incubated in the open laboratory for 85 days. This is mentioned merely as a possible explanation of the large amount of nitrites found in the checks. The results are given in tables XI and XII.

The checks in this series contained an unusual amount of nitrites. There are two possibilities which might explain this condition: (1) the cultures were incubated in the large laboratory where students were working and they might therefore, have absorbed the nitrites from the air; (2) it might have been in the chemicals or the water. The writer has no explanation for it and was surprised to find such a large amount present when the analyses were made.

The oxidation in this series was slower than in the one reported in table X, except in the case of the higher dextrose concentrations. Here the long incubation period brought about oxidation. The growth in general was in proportion to the dextrose content, see Plate III, Numbers 1, 2 and 3.

The growth in the nitrite cultures was similar to those with ammonium sulfate as shown in Plate III, 4, 5 and 6. These cultures were also incubated in the large laboratory as the others for 85 days. They were
TABLE XI

The Effects of Various Concentrations of Dextrose on the Oxidation of Ammonium Sulfate by Actinomyces GOO

<table>
<thead>
<tr>
<th>Number</th>
<th>dextrose</th>
<th>:Percent</th>
<th>Nitrite : nitrogen</th>
<th>Amount : nitrified</th>
</tr>
</thead>
<tbody>
<tr>
<td>check</td>
<td>none</td>
<td></td>
<td>0.417</td>
<td>0.568</td>
</tr>
<tr>
<td>check</td>
<td></td>
<td>&quot;</td>
<td>0.987</td>
<td>1.041</td>
</tr>
<tr>
<td>1</td>
<td></td>
<td>&quot;</td>
<td>1.041</td>
<td>1.014</td>
</tr>
<tr>
<td>2</td>
<td>0.01</td>
<td>1.561</td>
<td>1.801</td>
<td>1.309</td>
</tr>
<tr>
<td>3</td>
<td>0.05</td>
<td>3.750</td>
<td>3.435</td>
<td>2.933</td>
</tr>
<tr>
<td>4</td>
<td>0.10</td>
<td>3.946</td>
<td>3.675</td>
<td>3.183</td>
</tr>
<tr>
<td>5</td>
<td>1.00</td>
<td>8.090</td>
<td>6.930</td>
<td>6.438</td>
</tr>
<tr>
<td>6</td>
<td>2.00</td>
<td>7.739</td>
<td>7.753</td>
<td>7.261</td>
</tr>
</tbody>
</table>

* This column is obtained by subtracting the average of the checks from the average of the duplicates.
TABLE XII

The Effects of Various Amounts of Dextrose on the Oxidation of Nitrites by Actinomyces 600.

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>check</td>
<td>&quot;</td>
<td>16.24</td>
<td>13.44</td>
<td></td>
</tr>
<tr>
<td>check</td>
<td>&quot;</td>
<td>10.64</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>&quot;</td>
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<td>4.20</td>
<td>-9.24</td>
</tr>
<tr>
<td>1</td>
<td></td>
<td>7.00</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>0.01</td>
<td>8.96</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>0.01</td>
<td>12.88</td>
<td>10.92</td>
<td>-2.52</td>
</tr>
<tr>
<td>3</td>
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<td>12.60</td>
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<td>3</td>
<td>0.05</td>
<td>24.92</td>
<td>18.76</td>
<td>5.32</td>
</tr>
<tr>
<td>4</td>
<td>0.10</td>
<td>16.52</td>
<td></td>
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</tr>
<tr>
<td>4</td>
<td>0.10</td>
<td>22.96</td>
<td>19.74</td>
<td>6.30</td>
</tr>
<tr>
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<td>1.00</td>
<td>13.72</td>
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<td>1.00</td>
<td>58.80</td>
<td>36.26</td>
<td>22.22</td>
</tr>
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<td>6</td>
<td>2.00</td>
<td>68.32</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>2.00</td>
<td>48.40</td>
<td>55.86</td>
<td>42.22</td>
</tr>
</tbody>
</table>

* This column is obtained by subtracting the average of the checks from the average of the duplicates.
analyzed as outlined in Experiment 8, B. Table XII gives the results.

There is no agreement between the checks in this table. For this reason little weight can be given to the results. However, with 2 percent dextrose the oxidation is so large that it can hardly be due to error. The table would seem to indicate an error in analysis, but the writer has not been able to find any errors there. Perhaps the most that can be said is that this organism grows as well with nitrite nitrogen as with ammonia and may bring about considerable oxidation under favorable conditions.

Experiment 12.

In order to test the effects of various concentrations of dextrose on nitrification of ammonia by Actinomyces 200 and Actinomyces 300, the following experiment was carried out. Cultures were prepared in the usual way.

A. After inoculation with a suspension of the organisms the cultures were incubated at 30 degrees centigrade for 93 days. Table XIII shows the results.

There was little oxidation in any case as shown by this table. The growth appeared to be in
<table>
<thead>
<tr>
<th>Number</th>
<th>Percent</th>
<th>Nitrite N.</th>
<th>Average</th>
</tr>
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<tbody>
<tr>
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<td>0.082</td>
<td>0.061</td>
</tr>
<tr>
<td>Check</td>
<td>0.00</td>
<td>0.041</td>
<td></td>
</tr>
<tr>
<td>A. 200</td>
<td>0.00</td>
<td>0.162</td>
<td>0.208</td>
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<td></td>
<td>0.00</td>
<td>0.254</td>
<td>0.147</td>
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<td>0.083</td>
<td>0.063</td>
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<td>0.05</td>
<td>0.162</td>
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<td></td>
<td>0.05</td>
<td>0.083</td>
<td>0.061</td>
</tr>
<tr>
<td></td>
<td>0.10</td>
<td>0.054</td>
<td></td>
</tr>
<tr>
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<td>0.10</td>
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<td></td>
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<td>1.00</td>
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</tr>
<tr>
<td></td>
<td>2.00</td>
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</tr>
<tr>
<td></td>
<td>2.00</td>
<td></td>
<td></td>
</tr>
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<td>0.202</td>
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<td>0.01</td>
<td>0.115</td>
<td>0.097</td>
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<td>0.036</td>
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<td>3</td>
<td>0.05</td>
<td>0.083</td>
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<tr>
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<td>-------</td>
<td>-------</td>
</tr>
<tr>
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</tr>
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<td>2.00</td>
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<td>0.050</td>
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</table>

* This column is obtained by subtracting the average of the checks from the average of the various duplicates.
proportion to the amount of dextrose present but was chiefly on the bottom of the flasks. The checks do not agree and show considerable nitrite content, especially considering that these cultures were incubated in a closed chamber at a constant temperature.

Unlike Actinomyces 600, these organisms did not show any nitrites where 1 and 2 percent dextrose was used. Furthermore the oxidation was much lower than shown in other experiments with these organisms except in the absence of dextrose. In these cases, the highest results were secured with both organisms in this experiment.

**Experiment 13.**

It was thought that probably these organisms would have either a beneficial or harmful effect on each other if grown together. To test this proposition, a number of cultures were prepared in the regular way. Suspensions of organisms were used for inoculation 1 c.c. being used for each. Where two organisms were introduced only one-half c.c. of the respective suspensions was added making the total inoculum about the same in all cases. The cultures were incubated in the open laboratory for 45 days. Several checks were prepared to
try to learn what conditions favor absorption of nitrites from the air. These were treated as follows: Checks (a) and (b), Medium 1 without MgCO₃ in well stoppered flasks; (c) and (d) the same as (a) and (b) except MgCO₃ added; (e) and (f) Medium 1 without MgCO₃, plugged with cotton and left on the laboratory desk; (g) and (h), Medium 1 with MgCO₃ plugged with cotton and placed with the cultures; (i) and (j), Medium 1 with MgCO₃ and 0.10 percent dextrose, plugged with cotton and placed with the cultures. Table XIV gives the results after 45 days.

This series of checks is interesting. When well stoppered no nitrites appeared in the flasks either with or without MgCO₃. Where only cotton plugs were used and no MgCO₃ added, 0.03 p.p.m. of nitrite nitrogen was absorbed, and similar flasks to which MgCO₃ was added 0.078 p.p.m. of nitrite nitrogen was absorbed. Dextrose did not affect the rate of absorption either with or without MgCO₃. It is seen from these tests which check with those reported by Jordan and Richards (25) that great caution is essential in making qualitative tests as well as quantitative determinations of nitrites in incubated cultures.

In all but one case namely 35 and 36, the
TABLE XIV

The Effects of Dextrose on Certain Nitrifiers
When Growing Alone or Together.

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<th>Number</th>
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* This column is obtained by subtracting the average of the checks from the average of the various duplicates.
dextrose cultures gave higher results than similar cultures without dextrose, and in this case the difference is small. Actinomyces 500 and Actinomyces 600 are benefited by growing together both with and without dextrose. These organisms gave the highest single oxidation. Bacterium 500 grown with Actinomyces 200 did not oxidize as rapidly as when grown alone; but this is not the case when it was grown with Actinomyces 300 where a higher oxidation occurred than either organisms produced when grown alone.

**Experiment 14.**

In order to determine whether or not the six organisms described in this paper would nitrify ammonium sulfate in soil, the following experiment was carried out. Fifty gram portions of soil were weighed into 250 c.c. Erlenmeyer flasks and treated with 0.3 gram MgCO₃, and 15 mgm. nitrogen as ammonium sulfate. They were then made up to optimum moisture and sterilized at 18 pounds pressure for three hours in the autoclave. Half of the samples received 0.10 percent dextrose. Suspensions of the various organisms were used for inoculation.

The cultures were incubated at room temperature in the dark for 80 days. Sterile water was added every
two weeks to keep up the optimum moisture content, the amount needed being determined by weighing the flasks. The results are recorded in table XV.

In all but one case there was higher oxidation where no dextrose was added. This is probably due to the use of dextrose for energy by the organisms. There is a considerable soluble organic matter in autoclaved soil which may account for the small amount of nitrogen oxidized in most cases. The greatest oxidation was brought about by Actinomyces 600 in the absence of dextrose. This organism also showed the greatest difference between the soil treated with dextrose and that not treated. This experiment shows clearly that the organisms described in this paper are able to nitrify ammonium sulfate in soil as well as in solution.

MORPHOLOGICAL AND CULTURAL CHARACTERISTICS.

The morphological characteristics of actinomyces have been studied by several investigators including Krainsky (29), Drechsler (14), Waksman (52), and more recently by Orskov (41). All of these sources have been used for reference in these studies. The agar block as used by Orskov was found to be the most serviceable
TABLE XV

The Effects of 0.10 Percent Dextrose on Nitrification of Ammonium Sulfate in Carrington Loam by Certain Pure Cultures.

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for studying the germination of spores and colony formation. The aerial mycelia may be studied either in the colony or by removing to a slide, as suggested by Waksman (54) pages 291-292 and staining with the ordinary laboratory stains.

The cultural characteristics were studied by inoculating various media and recording the description of the growth produced. This is similar to the methods of Conn (13) and Waksman (52). However, the media has been chosen somewhat arbitrarily in these studies and it is not known what the value of some of these media is. This question can be settled only by a study of several known species.

Very few biochemical studies were made owing to lack of time. Starch hydrolysis and nitrite reduction were studied in solution and on agar plates. The other reactions have been recorded with the descriptions of growth.

**Culture Media**

Unless otherwise stated washed agar was used and the media were sterilized at 15 pounds pressure for 15 minutes. Distilled water was used in all media.

**Medium 1.** The modified Omeliansky solution
which has been described in preliminary Test 1. The only modification of this medium was the addition of various amounts of dextrose or starch.

Medium 2. This was described in Experiment 4. It was slightly modified by the addition of various amounts of dextrose.

Nutrient growth and nutrient agar. These were described in preliminary Tests I and II.

Gelatin. This was made by dissolving 150 grams of Bacto-gelatine in one liter of distilled water and tubing.

Dextrose agar (Krainsky's). K_2HPO_4, 0.5 gram; asparagin, 0.5 gram; dextrose, 10 grams; agar, 15 grams; water, 1 liter; pH, 6.8-7.0. (Krainsky used 30 grams dextrose per liter)

Calcium malate agar (Krainsky's). Calcium malate, 10 grams; NH_4Cl, 0.5 grams; K_2HPO_4, 0.5 gram; glycerin, 10 grams; agar, 15 grams; water, 1 liter; pH, 6.8-7.0.

Milk. Skimmed, tubed and autoclaved.

Litmus milk. Milk with litmus added.

Ammonium sulfate dextrose agar. (NH_4)_2SO_4, 1 gram; K_2HPO_4, 1 gram; NaCl, 0.5 gram; MgSO_4, 0.5 gram; K_2CO_3, 0.5 grams; dextrose, 10 grams; agar, 15 grams;
Bean agar. 300 grams white beans were cooked thoroughly in 1 liter of water and wrung through cheese cloth into a solution containing 10 grams dextrose and 15 grams washed agar. The residue was discarded and the volume made up to 1500 c.c. The agar was tubed and sterilized.

Starch agar. 10 grams of potato starch was suspended in 800 c.c. water and boiled down to 500 c.c. of Medium 1 having 0.25 gram K₂CO₃ substituted for the MgCO₃, and 15 grams agar were added. Then the medium was completed as the others.

Cellulose agar 1. 500 c.c. of cellulose suspension prepared according to McBeth and Scopes (35) was added to 500 c.c. of Medium 1 having 0.25 gram K₂CO₃ substituted for the MgCO₃. 15 grams agar were then added and the medium completed as the others.

Cellulose agar 2. 500 c.c. of cellulose suspension was added to 500 c.c. of a medium having the following composition: K₂HPO₄, 1 gm; MgSO₄, 0.5 gm; KCl, 0.5 gm; NaNO₃, 2 gms; CaCO₃, 2 gms; Fe₂(SO₄)₃, trace, water, 1 liter. This was then tubed and autoclaved.

Potato, carrot and turnip plugs. These were
prepared in the usual way and sterilized in autoclave.

Onion extract agar. 250 grams white onions were cut up into 250 c.c. water. They were cooked in the autoclave for 15 minutes at 10 pounds. This was filtered through a cloth, the volume made up to 500 c.c. and seven and one-half grams agar added. The medium was then tubed and completed as usual.

Humus agar. Peat was treated with one percent HCl and after standing a short time washed free from chlorides, four percent NH₄OH was then added and after 24 hours standing with occasional stirring, was filtered. The filtrate was allowed to stand for a few days and 100 c.c. was withdrawn and made up to 1 liter with distilled water. 15 gms. of agar were added and dissolved. The medium was then tubed and sterilized. The medium contained 12.60 p.p.m. nitrogen in the form of ammonia.

A number of media were made up to study the growth characteristics with different sources of carbon. The following carbon compounds were used and added in

* The author is indebted to Mr. A. O. Alben for preparing the humus used in this medium.
one percent concentration: Mannitol, lactose, d-galactose, levulose, xylose, mannose and sucrose. The basic solution was the same for all and contained the following constituents per liter: NaNO₃, 2 gms. K₂HPO₄, 1 gm. MgSO₄, 0.3 gm. KCl, 0.5 gm. Fe₂(SO₄)₃, trace. After dissolving 15 gms. of agar in one liter of this solution the various carbon compounds were added and the media completed as usual.

DESCRIPTION OF SPECIES

Actinomyces 200

I. Morphology.

1. Spirals.

None noted; hyphae long, slender, with profuse branching.

2. Conidia.

Spherical, forming chains which resemble streptococci. Usually four tubes formed when germinating.

3. Colonies: circular, see Plate VI.

II. Cultural Characteristics.

1. Medium 1 with 1 percent dextrose.
Growth: Usually white disc on surface showing concentric rings. If MgCO₃ is excessive, often no surface growth.

   Growth: None with or without dextrose.

3. Nutrient agar:

4. Nutrient broth
   Growth: Usually on the bottom of tube.
   Aerial mycelium. Abundant on surface growth when any occurs.
   Pigment: None.

5. Gelatin
   Growth: Very rapid at first, white to gray, slower after two or three days.
   Aerial mycelium. Few or none.
   Soluble pigment: Light yellow in liquefied portion.
   Liquefaction: Rapid at first, but not complete in six weeks.

6. Dextrose agar
   Growth: Yellowish-gray oily appearing,
very slightly raised. Most rapid in beginning.
Aerial mycelium: Develop slowly, white
Pigment: None.

7. Calcium malate agar
Growth: Brownish-gray, very rapid at first, wrinkled.
Aerial mycelium: White, abundant.
Pigment: yellowish-brown.

8. Milk
Growth: Slow; dark rim adhering to flask; on surface, yellowish-brown.
Aerial mycelium: few
Pigment: none at first, after digestion the liquid becomes light brown.

9. Litmus milk
Same as milk except liquid becomes darker brown.
Reaction: basic to litmus

10. Tyrosine agar
Growth: heavy pinkish-white, yellow globules of liquid abundant on growth.
Aerial mycelium: abundant, light pink.
Pigment: none

11. Ammonium sulfate dextrose agar
Growth: abundant yellowish growth
Aerial mycelium: white, abundant.
Pigment: light yellow

12. Starch agar
Growth: rapid, honey-like globules appear in 3 days. Surface growth becomes dirty yellow in 7 to 10 days;
Aerial mycelium: few, white, appearing around the edges of growth.
Pigment: none
Hydrolysis: rapid.

13. Bean agar
Growth: very rapid yellowish-brown, oily appearing
Aerial mycelium: begin around surface, rapidly covers all growth exposed, light pink.
Pigment: yellow, medium finally becomes light brown.

Growth: white, subsurface growth abundant, surface flat, radiating.
Aerial mycelium: few, white.
Pigment: none

15. Cellulose agar 2.
Growth: surface white flat radiating; subsurface, extensive.
Aerial mycelium: few, white.
Pigment: none

16. Potato plug
Growth: at first white; later dirty yellow to light brown, very wrinkled, raised.
Aerial mycelium: few, white.
Plug: turns brown.

17. Carrot plug
Growth: buff, slowly turns brown; very wrinkled.
Aerial mycelium: appear in week to 10 days, white at first; turn light pink; abundant.
Plug: brown.

18. Turnip plug
Growth: dirty yellow, barely raised, abundant.
Aerial mycelium: light pink, become very abundant.
Plug: brown

19. Onion extract agar: No growth
20. **Humus agar**
   Growth: slow, white, radiating from line of inoculation.
   Aerial mycelium: few, white
   Pigment: none could be detected

21. **Mannitol agar**
   Growth: white, fuzzy, radiating; subsurface well developed. After 1 month, faint pink, flat.
   Aerial mycelium: fine, quite numerous.
   Pigment: none.

22. **Lactose agar**
   Growth: slow at first, well raised. Subsurface well developed.
   Aerial mycelium: good development, white at first, becomes faint pink.
   Pigment: faint yellow

23. **d-Galactose agar**
   Growth: very good, subsurface well developed.
   Aerial mycelium: white at first, becomes pink.
   Pigment: light yellow.

24. **Levulose agar**
Growth: slow at first, well raised, profuse later.
Aerial mycelium: white at first, become pink and there appear many yellow globules of liquid which leave crater-like marks on the surface of growth.
Pigment: light yellow.

25. Zylose agar
Growth: slow at first, subsurface well developed.
Aerial mycelium: pink, radiating from line of inoculation.
Pigment: light yellow.

26. Manose agar
Growth: very good, subsurface well developed.
Aerial mycelium: white at first, turn pink, contain numerous yellow globules which leave crater-like marks on the surface of growth.
Pigment: medium slowly darkens.

27. Sucrose agar
Growth: good, subsurface well developed.
Aerial mycelium: white fuzzy, turn pink.
Covered with globules which leave crater-
like marks on the surface of growth.

Pigment: light yellow.

Actinomyces 300

I. Morphology

1. Spirals
   Numerous, only one turn usually.
   Hyphae long slender, crooked, profuse branching, slightly tapering.

2. Conidia
   Spherical or slightly ovoid; resemble streptococci. 2 or 4 tubes formed when germinating.

3. Colonies: circular, see Plate VII

II. Cultural Characteristics

1. Medium 1. with 0.1 percent dextrose.
   Growth: gray flat colonies on surface; growth on bottom mostly.
   Aerial mycelium: gray
   Pigment: none, dirty yellow with higher concentrations of dextrose.

   Growth: none with or without dextrose.

3. Nutrient agar
Growth: good, raised center.
Aerial mycelium: profuse, gray, covered with minute droplets which leave crater-like marks.
Pigment: medium becomes brown and finally deep purple.

4. **Nutrient broth**
   Growth: on surface and bottom, irregular mass.
   Aerial mycelium: gray, powdery, when present.
   Pigment: turns broth brown.

5. **Gelatin**
   Growth: rapid at first, then becomes slower.
   Aerial mycelium: white
   Pigment: brown
   Liquefaction: rapid at first, then slow, not complete in six weeks.

6. **Dextrose agar**
   Growth: white, rapid; subsurface well developed; becomes brown in old cultures.
   Aerial mycelium: few, develop very slowly gray.
   Pigment: medium slowly turns brown.
7. **Calcium malate agar**
   Growth: very rapid; pinkish-brown, wrinkled.
   Aerial mycelium: develop slowly, gray dusty appearance.
   Pigment: none, old cultures darken slightly.

8. **Milk**
   Growth: good, brown rim adhering to tube; mass on surface.
   Aerial mycelium: few, gray.
   Pigment: brown, medium looks like coffee and cream.
   Reaction: acid to litmus; digestion of milk very slow.

9. **Litmus milk**
   Growth: same as in milk.

10. **Tyrosine agar**
    Growth: good, not raised; subsurface well developed.
    Aerial mycelium: gray, radiating, dusty.
    Pigment: medium slowly darkens.

11. **Ammonium sulfate dextrose agar**
    Growth: white in young cultures, pinkish-brown in old; wrinkled; oily appearance;
12. Starch agar
Growth: rapid, flat; subsurface well developed.
Aerial mycelium: gray; dusty.
Pigment: none.
Hydrolysis: Rapid.

13. Bean agar
Growth: very rapid; buff; under surface of growth red;
Aerial mycelium: bluish-gray abundant
Pigment: brown

Growth: good; flat, subsurface well developed.
Aerial mycelium: white, fuzzy
Pigment: none.

15. Cellulose agar 2
Same as on cellulose agar 1.

16. Potato plug
Growth: very rapid; wrinkled; gradually covering all the plug.
Aerial mycelium: profuse, white.
Plug: black border around growth; whole plug black later.

17. **Carrot plug**
Growth: very heavy buff to brown; spreads over surface rapidly, wrinkled.
Aerial mycelium: light gray, dusty, few.
Plug: darkened.

18. **Turnip plug**
Growth: very rapid, spreads over all surface.
Aerial mycelium: light gray, dusty.
Plug: brown.

19. **Onion extract agar**
Growth: rapid, buff, spreading over surface, produces strong onion odor in 2-3 days.
Aerial mycelium: blue-gray; profuse.
Pigment: brown; medium all colored.

20. **Humus agar**
Growth: slow, but definite
Aerial mycelium: white, few
Pigment: none could be detected.

21. **Mannitol agar**
Growth: very heavy
Aerial mycelium: white at first, turns to blue-gray. Many liquid globules form which leave crater-like marks on surface.
Pigment: medium turns first yellow, then light brown.

22. Lactose agar
Growth: very heavy
Aerial mycelium: white at first, turns to blue-gray. Many liquid globules form which leave crater-like marks on surface.
Pigment: agar slowly darkens.

23. d-Galactose agar
Growth: good
Aerial mycelium: gray at first, later, blue-gray; many liquid globules form which leave crater-like marks on surface.
Pigment: medium turns yellow.

24. Levulose agar
Growth: slow, well raised; bubbling well developed.
Aerial mycelium: blue-gray.
Pigment: slightly darkened medium.

25. Xylose agar
Growth: slow at first, good later; subsurface, well developed.
Aerial mycelium: White, turns blue-gray; abundant.
Pigment: turns medium light brown.

26. **Mannose agar**
Growth: good
Aerial mycelium: white at first, turns to blue-gray; droplets of liquid form which leave crater-like marks.
Pigment: medium slowly darkens.

27. **Sucrose agar**
Growth: very good
Aerial mycelium: white at first, turns blue-gray, globules of liquid form which leave crater-like marks on the surface.
Pigment: medium slowly darkens.

**Actinomyces 400**

I. **Morphology**

1. **Spirals**
   None noted; hyphae short sharply tapering on most media.

2. **Conidia**
   Form in long thin threads; 0.4 micron by
0.8 micron; show granules when stained.

3. Colonies: round or oblong. See Plate VIII.

II. Cultural Characteristics

1. Medium 1. With 0.1 percent dextrose
   Growth: spherical or flat colonies form on surface; some growth on bottom.
   Aerial mycelium: gray
   Pigment: pink, darkens with age of culture.

2. Medium 2. With 0.1 percent dextrose
   Growth: very good on surface; some on bottom.
   Aerial mycelium: gray
   Pigment: pink, darkens with age of culture.

3. Nutrient agar
   Growth: rapid; dull gray, slightly raised.
   Aerial mycelium: gray, globules of liquid form which leave crater-like marks on surface.
   Pigment: none.

4. Nutrient broth
   Growth: sponge-like growth on bottom; seldom any surface growth.
Aerial mycelium: none, generally.
Pigment: none, broth remains clear.

5. **Gelatin**
   Growth: none

6. **Dextrose agar**
   Growth: very slow; white spots appear after 6 days which turn to blue-gray, oily appearing masses.
   Aerial mycelium: none
   Pigment: none

7. **Calcium malate agar**
   Growth: none

8. **Milk**
   Growth: slow at first, ring forms on tube near surface;
   Aerial mycelium: none noted.
   Pigment: milk becomes greenish when digestion is practically complete.
   Reaction: acid.

9. **Litmus milk**
   Growth: same as in milk
   Aerial mycelium: none noted.
   Pigment: after digestion, tubes become pink.
   Reaction: Acid
10. Tyrosine agar
Growth: slow, bluish-white, after 4 days.
Aerial mycelium: few, dusty, gray, only part of growth covered after 70 days.
Pigment: medium turns brown.

11. Ammonium sulfate dextrose agar
Growth: good; cream to white.
Aerial mycelium: white; numerous globules of liquid form leaving crater-like marks when they dry.
Pigment: none

12. Starch agar
Growth: good
Aerial mycelium: white fuzzy; numerous globules form which leave crater-like marks on surface.
Pigment: none
Hydrolysis: rapid

13. Bean agar
Growth: No growth

Growth: small pin point nodules appear in 4 days; these gradually enlarge; the sub-surface about equal to the surface growth.
Aerial mycelium: white
Pigment: none

15. Cellulose agar 2.
Growth: pin point dots appear in 6 days; these gradually increase in size.
Aerial mycelium: white
Pigment: none.

16. Potato plug
Growth: begins around margin after 2 weeks, white, gradually increases.
Aerial mycelium: white, powdery.
Plug: darkens.

17. Carrot plug
Growth: none

18. Turnip plug
Growth: slight after 2 weeks around edges of tube, gradually increasing
Aerial mycelium: white
Pigment: none

19. Onion agar
Growth: none

20. Humus agar
Growth: white spots develop in 3 days, increase in size.
Aerial mycelium: white
Pigment: none could be detected

21. Mannitol agar
Growth: slow, begins to be evident in week; raised; subsurface well developed.
Aerial mycelium: gray
Pigment: black; medium turns smoky.

22. Lactose agar
Growth: evident in week; surface well raised; subsurface well developed.
Aerial mycelium: white
Pigment: none

23. d-Galactose agar
Growth: evident in 4 days, blue gray.
Aerial mycelium: gray; powdery, numerous small globules form leaving crater-like marks on the surface.
Pigment: medium becomes pink, light purple, then dark, smoky

24. Lovulose agar
Growth: none

25. Xylose agar
Growth: evident in 2 weeks, develops well.
Aerial mycelium: gray; numerous globules
leaving crater-like marks on surface.

Pigment: none

26. Mannose agar

Growth: well raised, dirty white growth after one week; little subsurface development.

Aerial mycelium: white

Pigment: none

27. Sucrose agar

Growth: evident in 4 days; subsurface well developed.

Aerial mycelium: blue gray.

Pigment: medium becomes light purple under growth.

Actinomyces 600

I. Morphology

1. Spirals

None noted; hyphae usually thick, slightly tapering.

2. Conidia

Fine threads form which break up into rod shaped elements resembling bacilli; these are found to consist of small spherical
spores. As a rule one tube which branches profusely forms when germinating.

3. Colonies: oblong, deep when young, later, round, massive with hyphae extending slightly beyond the compact edge. See Plate XI.

II. Cultural Characteristics

1. **Medium 1**
   
   Growth: small floating masses appear in 4 to 6 days; surface white, dusty.
   
   Aerial mycelium: white, dusty
   
   Pigment: none

1a. Medium 1 with 0.1 percent dextrose
   
   Growth: upper surface white; under surface, yellow; on bottom and surface of liquid.
   
   Aerial mycelium: white, dusty
   
   Pigment: light yellow.

2. **Medium 2**
   
   Growth: white, lace-like, floating, appears in 4 to 7 days.
   
   Aerial mycelium: white.
   
   Pigment: none.

2a. Medium 2 with 0.1 percent dextrose.
-123-

Growth: white masses floating on surface, adhering to sides of flask; some on bottom. Aerial mycelium: white, dusty. Pigment: light yellow.

3. Nutrient agar

Growth: good, wrinkled, easily removed for there is scarcely any subsurface growth; yellow to brown. Aerial mycelium: few, white at first, turn to light pink. Pigment: one noted.

4. Nutrient broth

Growth: mass on surface; very little ever on bottom; very few subsurface mycelia; broth remains clear. Aerial mycelium: white Pigment: none

5. Gelatin:

No growth

6. Dextrose agar

Growth: heavy, shallow, no subsurface growth noted. Aerial mycelium: white, powdery; assumes a cream color in older cultures. Pigment: none
7. Calcium malate agar
Growth: tiny specks appear in 2 weeks; becomes well raised, pinkish; no subsurface growth.
Aerial mycelium: gray at first; later, light pink.
Pigment: none.

8. Milk
Growth: very slow; tiny white specks form in ring around tube in 2 weeks; gradually cover the surface, no coagulation noted.
Aerial mycelium: white.
Pigment: none
Reaction: basic

9. Litmus milk
Growth: begins after 2 weeks, gradually covers surface.
Aerial mycelium: white, dusty.
Pigment: milk turns light green in 4 weeks; white layer on bottom.
Reaction: basic

10. Tyrosine agar
Growth: thin surface layer, very little
subsurface development.
Aerial mycelium: white, become light pink.
Pigment: none.

11. Ammonium sulfate dextrose agar
Growth: shallow surface layer at first, dirty yellow; raised with wart-like protrusions, in old cultures.
Aerial mycelium: white, dusty.
Pigment: none.

12. Starch agar
Growth: poor, shallow surface growth
Aerial mycelium: white, fuzzy
Pigment: none
Hydrolysis: none

13. Bean agar
Growth: Pin point specks appear in 12 days; grow quite rapidly, form well raised pinkish growth.
Aerial mycelium: pink, dusty
Pigment: light yellow

14. Cellulose agar 1
Growth: growth in 4 days, shallow, white
Aerial mycelium: white, fine
Pigment: none
15. **Cellulose agar 2.**
   Like the growth on cellulose agar 1.

16. **Potato plug**
   Growth: none

17. **Carrot plug**
   Growth: none

18. **Turnip plug**
   Growth: small white specks adhering to tube near contact with plug in 7 days; slowly extend to water in bottom of tube, and after 3 weeks, attacks side of plug.
   Aerial mycelium: white
   Plug: no change in 6 weeks.

19. **Onion agar**
   Growth: none

20. **Humus agar**
   Growth: good in 3 days
   Aerial mycelium: white
   Pigment: none detected; medium is black

21. **Mannitol agar**
   Growth: pin point specks appear in 4 days; becomes wrinkled, well raised.
   Aerial mycelium: pink
   Pigment: none
22. **Lactose agar**
Growth: appears in 6 days as small specks; becomes smooth, surface and even edges.
Aerial mycelium: powdery white with pink tint.
Pigment: none

23. **d-Galactose agar**
Growth: appears in 4 to 6 days; develops well.
Aerial mycelium: whitish-pink, powdery
Pigment: none

24. **Levulose agar**
Growth: appears in 7 days as small dots; develops well; becomes heavy, wrinkled.
Aerial mycelium: light pink
Pigment: none

25. **Xylose agar**
Growth: develops well after 10 days
Aerial mycelium: light pink, powdery
Pigment: none

26. **Mannose agar**
Growth: small specks appear in 4 days; then develop well; becomes wrinkled.
Aerial mycelium: light pink; powdery.

Pigment: none

27. Sucrose agar

Growth: appears in 4 days; develops rapidly; becomes very heavy and wrinkled.

Aerial mycelium: light pink, powdery

Pigment: none

Bacterium 500

I. Morphology

**Form**: ovoid in stained preparations, more nearly a rod on agar block. See Plate IX.

**Size**: 1.4 micron by 2.0 to two and one-half microns in stained preparations; much larger when observed on agar block. Usually observed in chains of 2 to 6 cells or in groups. In stained preparations, resemble strings or bunches of sausages.

**Motility**: motile with one flagellum long, near pole; cells progress like train of cars in hanging drop.

**Staining reactions**: stains readily with ordinary stains; gram negative.

**Spore formation**: none noted.
II. Cultural Characteristics

1. Medium 1 with 0.1 percent dextrose
   Growth: liquid slowly becomes slightly turbid; the MgSO₄ becomes somewhat granular, like crumbs. No coloration, except with higher concentrations of dextrose.

2. Medium 2 with 0.1 percent dextrose
   Growth: none

3. Nutrient agar
   Growth: colonies develop rapidly, pink; in old cultures the growth becomes almost black.
   Medium: no change

4. Nutrient broth
   Growth: very rapid, chiefly on bottom of tube, when disturbed, white flakes become dispersed in the liquid, the broth rapidly clouding.

5. Gelatin
   No growth

6. Dextrose agar
   Growth: good in 24 hours; brownish-white
   Medium: No change

7. Calcium malate agar
Growth: good in 24 hours; whitish-gray; this slowly turns brownish-pink.
Medium: no change

8. **Milk**
Growth: apparent in 24 hours on surface in 4 weeks milk completely digested.
Liquid yellow to brown.
Reaction: basic

9. **Litmus milk**
Growth: Same as in milk at beginning; white layer of liquid forms on bottom. As digestion proceeds, the liquid becomes light yellowish-brown, then finally assumes a purplish tint.
Reaction: basic

10. **Tyrosine agar**
Growth: honey-like globules form in 8 to 10 days; become flat, blue-gray.

11. **Ammonium sulfate dextrose agar**
Growth: bluish-gray, honey-like globules.
Medium: No change

12. **Starch agar**
Growth: Appears in 2 to 3 days, blue gray.
Hydrolysis: complete in 2 weeks.
13. **Bean agar**
Growth: very rapid, covering line of inoculation in 15 hours, buff, oily appearance.
Medium: becomes light yellow.

14. **Cellulose agar 1**
No growth

15. **Cellulose agar 2**
No growth

16. **Potato plug**
Growth: honey-like globules appear in 2 days, this turns to buff, well raised, oily growth in 5 to 6 days. The growth darkens to a brown in 4 weeks.
Plug: darkens

17. **Carrot plug**
Growth: grayish mucous-like in one tube, nothing in other. Repeated with same result. Inconclusive.

18. **Turnip plug**
Growth: very rapid; buff, flat, rapidly covers all of plug.
Plug: brown

19. **Onion agar**
No growth
20. **Humus agar**  
No growth

21. **Mannitol agar**  
Growth: heavy buff and cream mottled, lobular; cream; turns to buff and buff to dark pink with age.  
Medium: no change

22. **Lactose agar**  
Growth: same as 21, medium darkens slightly.

23. **d-Galactose agar**  
Growth: brownish gray; becomes mottled buff and gray.  
Medium: darkens

24. **Levulose agar**  
Growth: same as 22, except medium becomes light yellow.

25. **Xylose agar**  
Growth: very poor, blue gray.  
Medium: No change

26. **Mannose agar**  
Growth: good brownish-gray at first, then very slow.  
Medium: no change
27. **Sucrose agar**

Growth: heavy brownish gray in 3 days; finally becomes dark pink in 4 weeks.

Medium: slightly darkened.

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**Coccus 800**

### I. Morphology

*Form*: coccus, some cells slightly ovoid in rapidly growing cultures.

*Size*: 0.6 to 0.8 micron average diameter.

Usually form sheets on the slide the cells being surrounded by a zoogleal mass. See Plate X.

*Motility*: could not detect, nor could any flagella be found.

*Staining reactions*: stains easily with ordinary stains. Gram negative.

*Spore formation*: none noted

### II. Cultural Characteristics

1. **Medium 1** with 0.1 percent dextrose

   Growth: chiefly on bottom, MgCO₃ slowly becomes granular crumby.

2. **Medium 2** with 0.1 percent dextrose

   Growth: on bottom, forming slimy mass

3. **Nutrient agar**
Growth: very rapid, white by reflected light, blue by transmitted. Subsurface colonies ovoid, granular, yellow to brown.
Medium: no change

4. **Nutrient broth**
   Growth: slow, forming long thin threads; if agitated, becomes cloudy.

5. **Gelatin**
   Growth: none

6. **Dextrose agar**
   Growth: very heavy blue gray
   Medium: unchanged.

7. **Calcium malate agar**
   Growth: very heavy bluish-white; after 24 days brown pin point spots appear on the surface of growth; these slowly enlarge.
   Medium: becomes brown

8. **Milk**
   Growth: rapid; becomes yellowish-brown, when digested.
   Reaction: basic

9. **Litmus milk**
   Growth: same as in milk; tubes become white on bottom, and after digestion pink.
   Reaction: basic
10. Tyrosine agar
   Growth: very heavy blue-gray, slowly turns to light brown
   Medium: becomes yellowish-brown

11. Ammonium sulfate dextrose agar
   Growth: blue gray; turns to light brown
   Medium: no change

12. Starch agar
   Growth: rapid; white with bluish tint.
   Hydrolysis: rapid.

13. Bean agar
   Growth: heavy creamy layer on surface
   Medium: gradually turns brown

   No growth

15. Cellulose agar 2
   No growth

16. Potato plug
   Growth: yellowish, creamy, spreads rapidly, gradually darkens.
   Plug: becomes brown

17. Carrot plug
   Growth: creamy, spreading rapidly; turns water in tube turbid.
Plug: one turned black; the other, brown

18. **Turnip plug**
   Growth: rapid, grayish-yellow; becomes light blue in 4 weeks.
   Plug: light brown.

19. **Onion agar**
   Growth: none

20. **Humus agar**
   Growth: none

21. **Mannitol agar**
   Growth: very good, gray white
   Medium: light yellow, then light brown

22. **Lactose agar**
   Growth: slow, bluish-gray, oily
   Medium: light brown

23. **d-Galactose agar**
   Growth: good, blue gray
   Medium: first, yellow; then light brown

24. **Levulose agar**
   Growth: good, blue gray
   Medium: light yellow

25. **Xylose agar**
   Growth: very slow; blue gray
   Medium: no change

26. **Mannose agar**
Growth: good, bluish-white
Medium: yellowish-brown

27. Sucrose agar
Growth: very rapid, blue gray
Medium: light yellow

STARCH HYDROLYSIS AND NITRATE REDUCTION

Medium 1 was prepared in the usual way and 50 c.c. portions placed in 500 c.c. Erlenmeyer flasks. To each was then added one-half gram of potato starch and the flasks sterilized. Sterile MgCO₃ was then added and the flasks inoculated.

At the same time a modified Czapek's solution was prepared containing K₂HPO₄, 1.0 gram; MgSO₄, 0.5 gram; KCl, 0.5 gram; FeSO₄, 0.01 gram; NaNO₃, 2 grams; water, 1 liter. This was divided into 50 c.c. portions in 500 c.c. Erlenmeyer flasks and one-half gram starch added to each. Those were sterilized and inoculated at the same time as the above set.

Organisms 200, 500, 400, 500 and 800 hydrolysed the starch in both solutions quite rapidly. They also reduced the sodium nitrate in the Czapek's solution giving a strong nitrite reaction in one to two
weeks. Bacterium 500 was the slowest in nitrate reduction and 800 the most rapid.

Actinomyces 600 grew very slowly in both solutions adhering to the sides of the flasks. It did not hydrolyse the starch in 6 weeks in either solution nor did it reduce the nitrate in the same time. There was a positive nitrite reaction in the ammonium sulfate flasks showing that some of the ammonia had been oxidized, or nitrites absorbed from the air. However, this reaction was not noted in the checks. Two checks of each medium were kept with the cultures and tested when the cultures were tested.

These observations confirm the belief of Leone (31) stated about 1887, that bacteria may successively oxidize ammonia or reduce nitrates according to the environmental conditions.

SYSTEMATIC POSITION OF THE NITRIFIERS.

The author believes that many of the soil organisms can utilize ammonia or nitrites as a source of energy under certain conditions. The organisms described by Winogradsky and others were in all probability very materially changed physiologically by
being cultivated in purely mineral solutions for considerable periods. That pure cultures were obtained is not unlikely but it is probable that either the majority of this group of organisms have not been isolated or their power to oxidize nitrogen compounds has not been ascertained.

In view of these considerations it is considered unwise to name the organisms described in this paper. The author intends to continue his studies on this group of organisms during the next year under the auspices of the National Research Council and hopes to discuss this subject after more data has been accumulated. At the present time the data is too meagre and conflicting to properly allocate the nitrifiers to their proper place in the scheme of classification.

SUMMARY

Preliminary tests are described which were carried out to try to isolate nitrifying organisms which would not grow in nutrient broth. These tests failed. All organisms isolated by means of silica gel or washed agar plates grew in nutrient broth.

Experiments were then conducted to learn if these organisms could multiply in a purely mineral
solution. It was found that they multiplied rapidly when transferred from an enrichment culture to a fresh mineral solution but if transferred from nutrient agar they died off rapidly. However, when a sufficiently large inoculum was used, the organisms survived and finally multiplied.

It was then decided to test the effects of a low concentration of dextrose on the multiplication of organisms transferred from nutrient agar to a mineral medium containing dextrose and it was found that the organisms not only multiplied rapidly but oxidized the ammonium sulfate in this mineral medium after four weeks. The next experiment confirmed these results and proved that these organisms can be cultivated in broth and can later nitrify ammonium sulfate when placed in a suitable medium. This shows that the broth test for purity of nitrifying cultures is worthless. While the organisms studied in this work grew in nutrient broth it is not at all certain that all nitrifiers would grow under these conditions.

Several tests are reported showing the effects of various concentrations of dextrose on the nitrification process in solution and in soil. No attempt was made to stimulate a high rate of oxidation, but
simply to demonstrate the ability of these organisms to nitrify ammonia and in some cases nitrites when placed in suitable environment. 100 c.c. portions of the medium in 500 c.c. erlenmeyer flasks were used for the cultures.

Morphological and cultural characteristics of the six organisms isolated are given. These species grow well on ordinary laboratory media, some growing better on one medium and some on another. Three of the species grow well with nitrite as the source of nitrogen but the others do not grow at all. Tests also show that these three organisms may oxidize either ammonium sulfate or nitrites depending on the conditions. However, when the three organisms are cultivated in an ammonium sulfate medium, very little nitrate appears. Five of the species hydrolyze starch rapidly and reduce nitrates.

These experiments indicate that ammonium sulfate and nitrites are used for energy only when more easily available sources of energy such as dextrose are lacking or limited. Further studies will be conducted on these organisms during the next year.
CONCLUSIONS

1. Six species of nitrifying organisms were isolated and studied in pure culture. Of these four were Actinomyces and two were bacteria.

2. These organisms grow well on most of the ordinary laboratory media, which shows that the broth test for purity of nitrifiers is worthless.

3. Tests are reported showing that these organisms will nitrify ammonium sulfate in soil and solution.

4. Morphological and cultural studies are reported showing some of the characteristics of these organisms.

5. Five of the organisms were found to reduce nitrates to nitrites or oxidize ammonium compounds depending on the environmental conditions.

6. This work seems to indicate that ammonia and nitrites are used for energy by microorganisms only when other more easily available sources are limited or absent.
During the course of this work the author has discussed his problem with Dr. P. E. Brown, Dean R. E. Buchanan, Dr. Paul Emerson, Dr. Fred Smith and Dr. C. H. Werkman. For their encouragement, helpful suggestions and criticisms he wishes to express his appreciation. He is especially indebted to Dr. P. E. Brown for reading and criticising the manuscript.
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Plate I.

A colony of Actinomyces 600 on silica gel from which this organism was originally isolated. Note the scars made by the micropipette when transfers were made. Enlarged about 3 diameters.
Plate II

Four colonies of Actinomyces on silica
gel, natural size. Organisms from these colonies
were isolated but not studied owing to lack of time.
Plate III

Actinomyces 600. From left to right on the picture the cultures are (1) medium 1 without dextrose; (2) medium 1 with 0.10 percent dextrose; (3) medium 1 with 2.00 percent dextrose; (4) medium 2 without dextrose; (5) medium 2 with 0.10 percent dextrose; (6) medium 2 with 2.00 percent dextrose.
Plate IV

Actinomyces 400. From left to right on the picture the cultures are (1) medium 1 with 0.10 percent dextrose; (2) medium 1 with 2.00 percent dextrose; (3) medium 2 with 0.10 percent dextrose; (4) medium 2 with 2.00 percent dextrose.
Plate V

Cultures of Actinomyces on soil. From left to right they are (1) Actinomyces 200 on soil with ammonium sulfate; (2) Actinomyces 300 on soil with ammonium sulfate; (3) Actinomyces 400 on soil with ammonium sulfate; (4) Actinomyces 400 on soil with sodium nitrite; (5) Actinomyces 600 on soil with ammonium sulfate; (6) Actinomyces 600 with sodium nitrite.
Plate VI

Colonies of Actinomyces 200 on nutrient agar stained with gentian violet. Photographed with 16 mm. objective, number 10 ocular.
Plate VII

Colonies of Actinomyces 300 on nutrient agar stained with gentian violet. Photographed with 16 mm. objective, number 10 ocular.
Plate VIII
Colonies of Actinomyces 400 developing on nutrient agar. Not stained. Photographed with 16 mm. objective, number 10 ocular.
Plate IX

Bacterium 500, a young culture, stained with gentian violet. Photographed with 1.9 mm. (oil immersion) objective, number 10 ocular.
Plate X

Coccus 800, a young culture, stained with gentian violet. Photographed with 1.9 mm. (oil immersion) objective, number 10 ocular.
Plate X
Plate XI

Colonies of Actinomyces 600 developing on nutrient agar. Not stained. Photographed with 16 mm. objective, number 10 ocular.