# OBSERVATIONS UPON THE ISOLATION OF NITRIFYING ORGANISMS.

I. L. BALDWIN and W. M. CROSS, Purdue University.

The oxidation of ammonia to nitrates by biological agencies is carried on in two distinct steps. Oxidation of ammonia to nitrites is accomplished by Nitrosomonas, a small short rod, and the oxidation of nitrite to nitrate by Nitrobacter, a longer and more slender rod.

Since the classical nitrification experiments of Winogradsky (5, 6, 7) in 1890, many investigators have devoted considerable time and thought to the problem of the isolation of the organisms responsible for these transformations. Impure cultures of the organisms capable of carrying on the chemical transformations have been secured with comparative ease in most cases. The problem of securing absolutely pure cultures of the organisms in question without the loss of their characteristic properties has proven to be a very difficult matter and has been attended with success in relatively few cases.

Impure or mixed cultures have commonly been secured by inoculating an appropriate medium with soil, watching the progress of the oxidation of ammonia to nitrite or nitrite to nitrate and transferring to fresh media when the oxidation has reached the maximum intensity. After several transfers, enrichment cultures prepared in this way show relatively few organisms other than the nitrifiers, but probably never become absolutely pure.

In order to secure pure cultures it is necessary to plate the organisms out and pick isolated colonies or to pick single individuals. Due to the nature of the growth of the organisms the picking of single individuals and subsequent multiplication is attended with great difficulties. Under laboratory conditions these organisms do not develop properly in the presence of any considerable amount of soluble organic matter, consequently the ordinary bacteriological solidifying agents can not be used in the preparation of plates of these organisms. Agar from which the soluble organic matter has been thoroughly washed, silica gels, and plaster of Paris blocks have been utilized in securing isolated colonies. Cultures secured by picking isolated colonies from these media commonly show a loss or very considerable diminution of the oxidizing power.

Tests for purity of the cultures include examination of the colonies developing upon solid media, microscopic examination, and testing for growth in beef broth. The nitrifiers will not develop in beef broth and the production of growth in such media is an indication of contamination with some organism other than the nitrifiers.

Warrington (1, 2, 3) and the Franklands (4) together with Winogradsky (5, 6, 7) were some of the early workers with this problem. Among the later workers the experiments of Beijerinck (8), Gibbs (10)

"Proc. Ind. Acad. Sci., vol. 34, 1924 (1925)."

and Bonazzi (9, 11, 12, 13) stand out pre-eminently. The work of Gibbs and Bonazzi present excellent reviews of the literature and no attempt will be made here to cover that phase of the problem.

In view of the difficulties encountered in the isolation of these organisms, it is felt that a statement of the technique employed and the results obtained in this laboratory may be of value.

**Technique.** The media used are in most cases slight modifications of those suggested by Gibbs and are very similar to those used by Winogradsky and Beijerinck.

The liquid medium for nitrite formation is as follows: Ammonium sulfate, 1.0 gram; di-potassium phosphate, 1.0 gram; sodium chloride, 1.0 gram; magnesium carbonate, 1.0 gram; magnesium sulfate, 0.2 gram; ferric chloride, trace; distilled water, 1000 cc.

The liquid medium for nitrate formation is as follows: Sodium nitrite, 1.0 gram; sodium carbonate, 1.0 gram; di-potassium phosphate, 0.5 gram; sodium chloride, 0.5 gram; magnesium sulfate, 0.3 gram; ferric sulfate, trace; distilled water, 1000 cc.

The solutions were prepared in bulk and then 25 cc. portions transferred to 150 cc. Erlenmeyer flasks which were sterilized in the autoclave at 15 pounds pressure.

For making solid media a 1.5 per cent agar was prepared by thoroughly washing the agar in several changes of distilled water, then dissolving by autoclaving and clarifying by filtering through cotton. The agar was then tubed in 10 cc. portions and sterilized in the autoclave. The following solutions were prepared and sterilized separately by adding the specified amounts of salts to 100 cc. of distilled water: A. Di-potassium phosphate, 1.5 grams; B. sodium carbonate, 1.5 grams, sodium nitrite, 1.5 grams; C. magnesium sulfate, 0.45 grams, sodium chloride, 0.75 grams, and ferric sulfate, 0.02 grams; D. ammonium sulfate, 1.5 grams, magnesium sulfate, 0.75 grams, ferric sulfate, 0.02 grams; E. sodium chloride, 3.0 grams, sodium carbonate, 1.5 grams.

In making Nitrobacter plates one cc. each of solutions A, B, and C were placed in sterile petri-dishes with sterile pipettes, the desired inoculum added to the melted and cooled agar and this poured into the plate, which was then thoroughly mixed. For the cultivation of Nitrosomonas one cc. each of solutions A, D, and E, plus the agar were used. To prevent the precipitation of the salts the one cc. portions of the various solutions should not be allowed to mix until the agar is added.

Tests for ammonia were made with Nessler's Reagent (14). Trommsdorf's Reagent (14) was used in testing for nitrites and Brucin Reagent (14) was used in testing for nitrates.

Isolations were made from a Miami silt loam and a Miami clay loam. The Miami clay loam was taken from a field under cultivation and was lighter in color and contained less organic matter than the other soil, which was taken from a blue grass meadow. The soil was taken from two depths, one from the upper six inches and the other between six and twelve inches.

The flasks containing the liquid media for the formation of nitrites and nitrates were each inoculated with one gram portions of soil from each of the sources and carried in duplicate. One cc. transfers were made to fresh flasks when tests showed sufficient oxidation. Such liquid cultures secured by inoculation with soil and carried in the enrichment process will be referred to as "crude cultures".

**Crude Cultures of Nitrobacter.** Table 1 records the progress of the oxidation of nitrite to nitrate by the crude cultures secured as previously described. Transfers were made in each case as soon as the cultures showed heavy nitrate production, and before all of the nitrite had disappeared.

From the data it is apparent that the variation between the different soils is no greater than the variation between duplicates. In fact after the first few transfers there is no apparent difference between any of the cultures.

The efficiency of the organisms was greater after the second generation in artificial media. The efficiency of the organisms had not decreased at the time the cultures were discontinued at the 16th generation, indicating that they might be carried indefinitely in such liquid cultures.

The length of time between the 8th and 9th generations is not due to any loss of efficiency in the organisms, but was the result of inability to make tests and transfers at the proper time.

TABLE 1. CRUDE CULTURE OF NITROBACTER

Number of days required for each transfer.

| | Miami Silt Loam | | | | Miami Clay Loam | | | |
|---|---|---|---|---|---|---|---|---|
| | Surface Soil | | Subsurface Soil | | Surface Soil | | Subsurface Soil | |
| | 1 | 1′ | 2 | 2′ | 3 | 3′ | 4 | 4′ |
| $F_0$ | 28 | 28 | 28 | 28 | 18 | 28 | 18 | 28 |
| $F_1$ | 10 | 10 | 10 | 10 | 17 | 10 | 17 | 10 |
| $F_3$ | 4 | 4 | 4 | 4 | 7 | 4 | 6 | 4 |
| $F_2$ | 3 | 3 | 3 | 3 | 3 | 3 | 4 | 3 |
| $F_4$ | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 |
| $F_5$ | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 |
| $F_6$ | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 |
| $F_7$ | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 |
| $F_8$ | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 |
| $F_9$ | 14 | 14 | 14 | 14 | 14 | 14 | 14 | 14 |
| $F_{10}$ | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 |
| $F_{11}$ | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 |
| $F_{12}$ | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 |
| $F_{13}$ | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 |
| $F_{14}$ | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 |
| $F_{15}$ | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 |
| $F_{16}$ | 8 | 8 | 8 | 8 | 8 | 8 | 8 | 8 |

**Pure Cultures of Nitrobacter.** To get the Nitrobacter organism isolated from other organisms, agar plates were made from each of the crude cultures. Two sets of plates were poured, one set was incubated at 37° C. while the other was incubated at room temperature, approxi-

mately 22° C. The plates were examined every day or two for colonies. After six days incubation colonies were visible upon the plates incubated at room temperature. These plates were examined under the low power of the microscope and the different colonies located. Two types of colonies were present, differing only in color, one yellow and the other white. These colonies varied in their outline from a fairly definite to a less definite form and were small and round.

In order to test these colonies one of each was removed from the plate and placed in liquid medium for nitrate formation. Four days later a test for nitrate showed its presence in each of the liquid cultures in considerable amounts.

Plates were again made from each of these flasks. In about eight days colonies were found developing. The colonies which developed resembled those from the first plating and were closely similar in appearance, varying from yellow to white in color. Several of these colonies were removed and placed on agar slants of the appropriate medium. These developed into long, scanty, whitish streaks.

To further test these organisms they were again placed in the liquid medium for nitrate formation, with the result that the nitrites were oxidized to nitrates.

The organism isolated is rounded and slightly longer than it is wide, being from .4 to .6 microns by .8 to 1.2 microns long. The surface colony varies in its outline from almost round to very irregular. The growth is denser near the center than at the edge, which has a tendency to be granular with a definite edge. The deep colony is oblong and more regular in outline than is the surface colony. Both the surface and deep colonies vary in color from almost pure white to a creamy yellow.

**Crude Cultures of Nitrosomonas.** The crude cultures of Nitrosomonas were secured and carried in the same way as those of Nitrobacter. Transfers were made in every case as soon as vigorous oxidation of the ammonia to nitrite occurred. The results of this experiment are recorded in Table 2, and the data obtained justifies the following statements.

The Nitrosomonas organism is much slower in its oxidation processes than is the Nitrobacter. Also the rate of oxidation varies considerably from time to time and with different cultures.

The efficiency of the organisms of one soil seems to be no greater than that of the others. The organisms in the duplicate flasks are as variable as the organisms of the different soils in their efficiency. In certain cases one of the two duplicates became inactive while the other retained its activity. No explanation can be given for this at present.

In those cultures remaining at the time the experiment was discontinued a slight increase in the speed of oxidation is noted in the last transfers when compared to the first transfers.

TABLE 2. CRUDE CULTURES OF NITROSOMONAS

Number of days required for each transfer.

| | Miami Silt Loam | | | | Miami Clay Loam | | | |
|---|---|---|---|---|---|---|---|---|
| | Surface Soil | | Subsurface Soil | | Surface Soil | | Subsurface Soil | |
| | 5 | 5′ | 6 | 6′ | 7 | 7′ | 8 | 8′ |
| $F_9$ | 14 | 14 | 14 | 14 | 14 | 14 | 14 | 14 |
| $F_1$ | 18 | 26 | 20 | 15 | 15 | 13 | 13 | 18 |
| $F_3$ | 24 | 35 | 25 | 19 | 11 | 27 | 27 | 16 |
| $F_2$ | 20 | 23 | 10 | 11 | 14 | 11 | 24 | 15 |
| $F_4$ | 12 | 23 | 9 | 20 | 13 | 20 | 20 | 17 |
| $F_5$ | 10 | 22 | 11 | 29 | 9 | 7 | ..... | 10 |
| $F_6$ | 12 | ..... | 11 | 14 | 10 | 11 | ..... | 21 |
| $F_7$ | ..... | ..... | ..... | ..... | 12 | 14 | ..... | ..... |

**Pure Cultures of Nitrosomonas.** Plate cultures were made of the Nitrosomonas organisms as in the case of the Nitrobacter and incubated at the two temperatures. After a period of ten days colonies were visible upon the plates incubated at room temperature. The colonies resembled very closely those of the Nitrobacter, varying from white to creamy yellow. Agar slants were prepared from these colonies. In about two weeks a thin light spreading growth was visible to the naked eye. Transfers from these to the liquid medium for nitrite formation showed oxidation of the ammonia to nitrite after a period of about two weeks. Oxidation in several cases appeared to be much slower than in the crude cultures of Nitrosomonas.

The organism isolated was round to oval in shape, varying from .5 to .8 microns in width and .6 to .9 microns in length. The colony on agar plates was slightly more irregular than the Nitrobacter colony, but otherwise resembled it very closely. The color varied from whitish to almost brown.

**Summary.** I. The enrichment culture process used did not seem to injure the oxidizing power of the Nitrobacter. However, in the case of Nitrosomonas more variable results were secured. Certain cultures lost their ability to oxidize ammonia while others seemed to gain in their oxidizing power.

II. Agar plates and slants were used with success in growing both Nitrobacter and Nitrosomonas. Cultures transferred from agar into liquid media still retained their power of oxidation.

III. Nitrobacter is much more rapid in its oxidation processes than is Nitrosomonas when carried in pure or relatively pure cultures.

IV. The kind of soil and the depth from which the samples were taken seemed to exert no permanent effect upon the oxidizing efficiency of the nitrifiers.

## LITERATURE CITED.

1. Warrington, R. Jour. Chem. Soc. Vol. 33, pp. 44-51; Vol. 35, pp. 429-456; Vol. 45, pp. 637-672. 1878-1884.

2. Warrington, R. Chem. News, Vol. 61, p. 135. 1884.

3. Warrington, R. Trans. Chem. Soc., Vol. 59, pp. 484-529. 1890.
4. Frankland, Grace C. and P. E. Trans. Roy. Soc., Ser. B, Vol. 18, pp. 107-128. 1890.
5. Winogradsky, M. S. Ann. Pasteur Inst. t. 4, pp. 213-257, 760-771. 1890.
6. Winogradsky, M. S. Ann. Pasteur Inst. t. 5, pp. 577-616. 1891.
7. Winogradsky, M. S. Compt. Rend. Acad Sci. t. 113, pp. 89-92. 1891.
8. Beijerinck, W. Folis Microbiol. Jahrg. 3 Heft, pp. 91-113. 1914.
9. Allen, E. R. and Bonazzi, A. Ohio Agr. Exp. Sta. Tech. Bul. 7. 1915.
10. Gibbs, W. M. Soil Science, Vol. 8, Dec., 1919.
11. Bonazzi, A. Jour. Bacty. Vol. 4, Jan., 1919.
12. Bonazzi, A. Jour. Bacty. Vol. 6, Sept., 1921.
13. Bonazzi, A. Jour. Bacty. Vol. 8, July, 1923.
14. Fred, E. B. Laboratory Manual in Soil Bacty. pp. 135-137.