SPECIAL ARTICLES

THE ISOLATION OF NITROSOMONAS AND NITROBACTER BY THE SINGLE CELL TECHNIQUE[1]

SINCE the classical work of Winogradsky in the late eighties, numerous attempts have been made to isolate the nitrifying organisms, but with few exceptions these have failed, and his work in its entirety has never been repeated. He used a modification of the plate method of Koch, and after overcoming many difficulties reported the isolation of both groups. The failures in isolation of these organisms have been due largely to the use of the plate method, a method which is not satisfactory for slowly growing organisms which do not readily form colonies.

These specialized organisms are usually cultivated in highly selective media and the cultures are called enrichments. For example, the only energy source in the medium for the study of the *Nitrosomonas* group is an ammonium salt and for the *Nitrobacter* group, sodium nitrite. Theoretically the bacteria which are unable to utilize these sources of energy should die out and those which are able to utilize the energy should increase in numbers. In a general way this is what happens, but it is not the whole story. If it were it would be an easy matter to make a few transfers followed by dilutions and in this way secure a pure culture of the desired form. In practice, however, all the dilution experiments have failed to yield pure cultures. As noted above, Winogradsky reported successful results by the use of the plate method and a few others have also reported isolations, but the vast majority have failed. This is due to the fact that most of the colonies which form on the plates contain other organisms which do not oxidize the ammonium salt or the nitrite. Furthermore, these colonies are microscopic and hard to study or to use for isolations. The criterion of purity (the failure to grow when inoculated into nutrient broth) is not satisfactory.

In the spring of 1928 the author began some studies on nitrifying organisms using the plate method. The results were not satisfactory. When the work was resumed in the following November, it was thought worth while to try the isolation of the organisms by picking single cells from the enrichment cultures. The first trials were very discouraging; it was slow and tedious to isolate organisms from the cultures having insoluble carbonates present which is the case in *Nitrosomonas* cultures. Of the first 365 cells isolated only thirty-four grew and none of them was found to oxidize the ammonium salt. This work shows, however, that non-oxidizing forms not only persist in the enrichments but that it is possible for them to develop from single cells in a mineral solution. It was clear that the enrichments must be improved, *i.e.*, the proportion of nitrifiers to other forms increased, if the method was to be successful. If some chemical could be found which is not toxic to the nitrifiers and which at the same time would materially retard the growth of other soil forms in the cultures, a satisfactory enrichment for isolations would be secured. Some of the dyes were tried for this purpose on the *Nitrosomonas* group but they were found to be more toxic to the nitrifier than to the contaminating forms in the cultures. Other chemicals were tried but without success. Finally copper carbonate when used with an equal amount of calcium carbonate was found to reduce the numbers of organisms which develop on a nutrient agar plate (nitrifiers do not develop on this medium) while it did not stop the oxidation. From enrichments prepared with this compound only some twenty cells were picked before a culture was secured which would oxidize the ammonium salt as rapidly as the enrichment from which it was isolated.

Fifty-six cells were isolated from the first pure culture, and of these two developed and oxidized the ammonium salt as rapidly as the mother culture.

In the case of the *Nitrobacter* group (those which oxidize the nitrites to nitrates) it was found recently by Prouty[2] that they could tolerate certain dyes. This treatment ought to reduce the numbers of contaminating forms in the cultures but there are as yet insufficient data on the subject. Enrichments were prepared in the usual way, that is, by transferring to fresh sterile medium when all the nitrites had disappeared from the cultures. After the fourth transfer since starting with soil, the cultures were exposed to a 1 per cent. solution of rosanaline hydrochloride for ten minutes, inoculated into fresh sterile medium and set aside for ten to fifteen days. From such a culture 101 cells were picked, of which fifteen grew and thirteen oxidized the nitrites as rapidly as the enrichment from which they had been isolated. Here it should be noted that two cells developed in the mineral solution which would not oxidize the nitrite. This is further evidence that non-oxidizing forms readily develop in the cultures. An average of twenty-two cells were isolated per day, but this does not include the time required for making media and preparing the apparatus for isolations.

[1] This work was made possible by the National Research Council and carried out in the laboratories of agricultural bacteriology, University of Wisconsin. The author is indebted to Dr. E. B. Fred for helpful suggestions and criticisms during the course of the work.

[2] Chas. C. Prouty, "The Use of Dyes in the Isolation of a Nitrite-oxidizing Organism," *Soil Sci.*, 28: 125–136, 1929.

Reisolations by picking cells from one of the pure cultures were made. Twenty cells were isolated and ten of them grew and oxidized the nitrites. If the percentage of cells which grow is the same in the isolations from crude cultures as from pure cultures it would indicate that only 30 per cent. of the cells in the enrichments were nitrite-oxidizers. The results are very satisfactory since it required only a few days to secure these cultures.

From this brief discussion it is seen that it is possible to secure pure cultures directly from enrichments by isolating single organisms; and when all the factors are considered, this method is more satisfactory for the nitrifiers than the plate method which does not usually yield pure cultures and which requires a great deal of time for carrying out.

Details of the method used and descriptions of the organisms isolated will soon be published.

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THE BORON CONTENT OF ORANGES

IN connection with a study of the relation between the boron content of irrigation water and a certain type of injury to citrus and walnut trees in southern California, it has been observed that these plants take up substantial quantities of boron which is deposited in the leaves. This fact has made it possible to diagnose cases of boron injury. Boron occurs as a natural constituent of practically all irrigation waters in southern California. In most of these waters the content of elemental boron ranges from .15 to .3 parts per million, while in those supplies that have caused trouble the boron content may range up to 2.5 parts per million. Orange trees irrigated with low-boron water have in their mature leaves from 50 to 150 parts per million of boron, based on the dry weight of the leaves. Those irrigated with high-boron water may show from 600 to 1,000 parts per million of boron to the dry weight of the leaves.

A very few analyses of fruits and of twigs or chlorophyll-bearing branches, chiefly of lemons, have indicated that most of the boron taken up by these trees is deposited in the leaves, but it has seemed worth while to ascertain whether or not there are appreciable differences in the boron content of the fruits produced by trees having pronounced differences in the boron content of their leaves. For this purpose a number of analyses have been made of fruits of navel orange trees grown under different conditions with respect to the boron content of the irrigation water and known to have very different proportions of boron in the leaves. In view of the fact that the skin of the orange is green when the fruit is young, it seemed desirable to make separate analyses of the skin and the pulp.

The fruits to be analyzed were picked from the trees in the early part of March, 1929, when they were fully mature. At the same time samples of mature leaves were picked from the same trees. The boron content of the irrigation water used in each grove had been determined previously by analyzing a series of samples taken at different times. In preparing the fruits for analysis the skins were removed and the pulp was dried in the presence of sodium and calcium hydroxides to prevent the loss of any boron by volatilization. The peel was dried without treatment. When the fruit material was thoroughly dry it was ground to a fine meal and the boron content was determined by the Chapin method, as modified and developed by Wilcox in the Limoneira Laboratory. The leaves were analyzed by the same method.

The essential results of these analyses are given in the accompanying table. Samples 1 and 2 were obtained from the vicinity of Riverside, California, where the boron content of the irrigation water is low. Samples 3 and 4 were obtained in the Santa Clara Valley where some of the irrigation waters have a high boron content. The boron content of the

THE BORON CONTENT OF IRRIGATION WATER AND OF THE LEAVES AND FRUIT OF NAVEL ORANGES IRRIGATED WITH THE WATER

	No. 1	No. 2	No. 3	No. 4
Irrigation water, boron, parts per million	.20	.35	1.25	2.45
Leaves, boron, parts per million	35	90	515	854
Ave. fresh wt. per fruit, grams:				
Pulp	133.2	95.6	119.3	141.7
Peel	61.9	44.0	69.6	61.3
Ave. dry wt. per fruit, grams:				
Pulp	17.0	13.6	13.6	15.3
Peel	14.8	12.0	15.0	12.5
Boron content, parts per million:				
Dry pulp	10	11	22	38
Dry peel	21	22	40	44
Boron content per fruit, mgs:				
Pulp	0.17	0.15	0.30	0.58
Peel	0.31	0.26	0.60	0.55
Whole fruit	0.48	0.41	0.90	1.13
As boric acid, per fruit, mgs:				
Pulp	0.97	0.86	1.71	3.31
Peel	1.77	1.48	3.43	3.14
Whole fruit	2.74	2.34	5.14	6.45