A STUDY OF THE ADAPTABILITY OF THE WINOGRADSKY PLAQUE METHOD FOR THE DETERMINATION OF THE MINERAL NUTRIENT CONTENT OF A VIRGINIA SOIL

A Thesis

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By

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Approved:

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TABLE OF CONTENTS

Table of Contents -2-

HISTORICAL

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The depletion of the native fertility of soils has made the application of commercial fertilizers an important prerequisite to successful crop production on many soils. Recommendations for fertilizing practices have been based largely on field tests and the response of plants to the series of variously treated plots is taken as an indication of the nutrient needs of the soil. These tests are both time consuming and expensive. They are directly applicable only to the soil on which the test is made and under the particular climatic conditions prevailing during the time the test was being carried on.

It is generally accepted that the field test is not a satisfactory basis for recommendations for fertilizing practices to farmers who have soils of widely varying physical and chemical characteristics. In an attempt to find a satisfactory method by which individual soils can be rapidly typed as to relative fertility, numerous tests have been proposed.

With the rise in soil bacteriology and methods for the study of the soil microflora, workers have tried to find an index of fertility in the growth and activity of these organisms. A study of the response of the soil

microorganisms to a treatment suggested for use under field conditions provides a clue to the probable response of the higher plants in a relatively short period of time.

The numbers of microorganisms present in a soil has been studied as a possible index of fertility. Waksman and Starkey (35) report data showing a marked variation between the number of microorganisms in plots that had received various fertilizer treatments. In a general way it can be said that the number of microorganisms present in the soil varies with fertility, in that soil conditions determine the number of organisms present. The soil reaction, the presence of organic matter, and minerals all influence the number of bacteria present.

The ability to convert ammonia into nitrates is more or less common to all soils. Several methods have been developed to measure the nitrification power of the soil (35), p. 715. A definite correlation has been found between this ability and crop productivity (9), (13), and (34). Lipman and Burgess (14) and Fraps (8) have used the nitrifiability of nitrogenous fertilizers on various soils as an index of the availability of these materials.

Russell (20) observed that the oxygen absorbed by a soil varied with fertility. Van Suchtelen (33) studied the carbon dioxide evolution of soils and concluded that it was a more accurate index of bacterial activity in the soil than the bacterial count. As all living bodies give off carbon dioxide, its evolution is an expression of all biological activity within the soil. Waksman and Starkey (37) report that carbon dioxide evolution can be used in grading soils on the basis of their fertility.

Christensen (1) suggested that the cellulose decomposing power of the soil may serve as an index to fertility. Mutterlein (16) placed pieces of filter paper weighing 10 grams at various depths in the soil and weighed the residue. He took the loss in weight of the filter paper as the cellulose decomposing power of the soil.

Niklewski (19) added cellulose to the soil and measured the carbon dioxide evolution. He found that the presence of available nitrogen controlled cellulose decomposition.

The ability of a soil to liberate oxygen from hydrogen peroxide has often been found to vary with fertility. Waksman and Dubos (36) found catalase

activity of soils to vary with the number of microorganisms present, certain organic and inorganic constituents, and other factors. They concluded that the phenomenon was complex and could not be used as a method of determining soil productivity, unless the various factors were determined individually.

As the bacterial count, carbon dioxide evolution, and nitrification are definite functions of the activity of the soil microflora, studies have been made to determine the relationship between crop productivity, fertilization, and these factors. Russell and Appleyard (21) found these factors to be interrelated. A rise in bacterial numbers was accompanied by a rise in carbon dioxide in the soil air, and somewhat later by a rise in the nitrate content. Plate I shows a graphical representation of the correlation between crop yields and microbiological activities on a series of variously fertilized plots.

The "A" plots were unlimed and the "B" plots were limed once every 5 years with 5 tons of ground limestone per acre; plot 5 received manure and minerals (640 lbs. superphosphate and 320 lbs. of potassium chloride); plot 7, no fertilizer; plot 9, sodium nitrate (320 lbs. annually) and minerals as in plot 5; plot 11, ammonium sulfate equivalent to the sodium nitrate applied

to plot 9 and the same minerals.

This graph clearly shows that microbiological activity is associated with fertilizing practices and crop productivity. The influence of lime is shown in plots 7 and 11. In the limed section of plot 11, four times as much nitrification occurred as in the unlimed section. The manure plots are outstanding because they show that high crop yields are definitely associated with an active microbiological flora.

The preceding discussion has been concerned with the action of the complete soil microflora or with a large group of these organisms in relation to soil fertility. A number of methods have been developed that use one specific genus or species of microorganisms to study the nutrient content of the soil.

Certain fungi have been used to supply information as to the phosphorus and potassium content of soils. This test is referred to in the literature as the Niklas Method.(18). Phosphorus or potassium free nutrient solutions are prepared, a certain amount of soil is added to each flask and inoculated with a fungus such as Aspergillus niger. After a suitable incubation period the fungus growth is removed, washed, dried and weighed. The growth is proportional to the amount of available

phosphorus or potassium present in the soil.

Smith, et al, (27) report a fair correlation between the Niklas (18) and the Truog methods (32). They used media containing one per cent citric acid; therefore the results actually show the one per cent citric acid soluble phosphorus. The Truog method uses 0.002 N.H_2SO_4 buffered to a pH of 3.00. The citric acid media used by Smith, et al, had a pH of 4.05. As the H-ion concentrations were so nearly the same, a fair agreement would be expected.

Truffant and Bezssonoff (31) report a bacteriological method for the determination of the available phosphorus in phosphatic fertilizers. They employed the principle used in the Niklas method. The phosphatic material to be tested was added to phosphorus-free nutrient medium. The sum of the nitrogen fixed by three organisms, an aerobe, a facultative aerobe, and an anaerobe, is taken as an index of the availability to the material tested.

Christensen (2) was probably the first investigator to employ the stimulation of the Azotobacter genus in soil fertility studies. He used it to determine the lime requirement. The test included two phases. One for the presence of Azotobacter and another for the

vigor of growth as measured by film formation in mannite solution. The former definitely typed the soil as to degree of acidity and the latter was an expression of the alkalinity and buffer capacity. Christensen (3) found soils in which phosphorus was the limiting factor for Azotobacter growth and entertained the hope that the relationships between the Azotobacter group, lime, and phosphorus content of soils could be used to supply information as to their productiveness.

What was probably a modification of Christensen's procedure has been used rather extensively to study the relative phosphorus content of soils. If the native acidity of the soil, and the acids produced by Azotobacter during growth are neutralized, growth is then proportional to the amount of available phosphorus present in the soil, if the other nutrients are supplied in liberal quantities.

A certain amount of soil is added to a phosphorus free mannitol nutrient solution containing 1 to 2 per cent calcium carbonate.

The materials are then sterilized and inoculated with a fresh culture of Azotobacter. After incubation, the atmospheric nitrogen fixed can be determined as an index of the phosphorus content of the

soil or the relative phosphorus content can be estimated, using the film formation on the nutrient solution as an index.

In 1926, Winogradsky (39) described the direct or spontaneous culture method for the study of the nitrogen fixing organisms of the soil. He added carbohydrate material and wetted the soil with water to a putty-like consistency. After incubation, large opalescent colonies of Azotobacter had appeared on some of the soils. Winogradsky and Ziemiecka (40) report that in soils that do not produce spontaneous colonies of Azotobacter, the addition of phosphates, lime or the two in combination usually allowed those colonies to develop. They concluded that the direct culture method could be used to study phosphorus, lime, and possibly other soil deficiencies. To this conclusion, the Winogradsky or Azotobacter plaque test owes its inception.

In principle the plaque test is similar to the field plot test. Various nutrients are added to a series of four plaques and Azotobacter colonies are produced in 72 hours. The number and vigor of the colonies on the fertilized plaque, when compared with those of the unfertilized plaque, give information as to the nutrient content of the soil being tested. The mineral nutrient

requirements of Azotobacter and farm crops are similar. Advantage is taken of this fact in estimating what might be normally expected if a crop is grown on the soil tested, and fertilized in a way analagous to that of one of the plaques.

The advantages of this method are the short time required and the simplicity of technic. A crop of Azotobacter colonies can be grown in 2 to 3 days that make certain nutrient deficiencies easily visible to the naked eye. Whereas the Neubauer method (6) requires a much longer period and intricate chemical analyses. With the field plot test, at least one growing season is necessary and a careful analysis of the data must then be made before conclusions can be drawn.

The Azotobacter or Winogradsky plaque test has been used by Ziemiecka (40), (41) to study Polish soils. Niklas (17) used it to study Polish soils and Guittonneau (11) to study soils from the plum orchards of France. These investigators found the method to be a satisfactory indication of phosphorus and lime deficiencies. In the United States, Sackett (22), (23), (24) reports the use of the method on Colorado soils. The beet sugar companies of the west became interested in it, and The Great Western Sugar Company alone is now testing 5000

soils annually by the plaque method, (4).

Workers in the laboratories of The Great Western Sugar Company have divided soils into five classes on the basis of their colony formation (15), (24). The classes correspond in a general way to those of the Neubauer method.

The names of the classes and the colony formation for each are as follows:-

Class 1. Not deficient. Neubauer classes 1 - 3, inclusive.

Colonies on both fertilized and unfertilized plaques approximately equal in number and development.

Class 2. Slightly deficient. Neubauer classes 4 and 5.

Unfertilized plaque -- Colonies as numerous as fertilized plaque, but smaller and less vigorous.

Fertilized plaque -- Colonies few to numerous, distinct and vigorous.

Class 3. Moderately deficient. Neubauer classes 6 - 8, inclusive.

Unfertilized plaque -- Colonies few to as many as fertilized plaque, but much smaller and

weaker in development; none approaching size of colonies on the fertilized plaque, pigment often less to none.

Fertilized plaque -- Colonies few to numerous, distinct and vigorous.

Class 4. Very deficient. Neubauer classes 9 and above.

Unfertilized plaque -- Colonies none or few to many, extremely small and feeble.

Fertilized plaque -- Colonies few to numerous, medium to large, distinct and vigorous.

Class 5. Doubtful.

Soils on which colonies fail to develop.

The only literature available on the use of the plaque test outside of the beet producing areas of the west, is a study of its adaptability to Ontario soils (12). Jones tested three groups of soils. Group I included 12 samples from check plots from fertilizer tests in the Province, and on which the response of crops to phosphorus fertilization was known. According to the test, this group of soils were all deficient in available phosphorus. As all of them had responded to phosphate fertilization, Jones concluded that there was a correlation between the plaque test and crop response.

Group 2 included 7 samples from a fertilizer experiment in Welland County. These soils had received applications of superphosphate or tricalcium phosphate in combination with lime, sulfur, or a complete fertilizer. The response of this group of soils to the plaque test indicated that they were all deficient in phosphorus. With the exception of the two check plots, all of the plots had received liberal applications of phosphorus. Jones explained this apparent discrepancy by assuming that the preceding crop had removed most of the available phosphorus from these soils. The available phosphorus as shown by the Truog method, with the exception of the plots receiving tricalcium phosphate, was as low as, if not lower, than the check plots. Jones concluded that the plaque test indicated that more phosphorus was necessary to give good returns for the next crop, and that there was a fair amount of correlation between the results of the plaque test and crop returns.

The third group included 2 samples of muck-swamp soil. One sample was taken from an area on which potatoes, onions, and lettuce had been grown for four years. It had been heavily fertilized with phosphorus. The available phosphorus by the Truog method was 413 p.p.m. It produced vigorous colonies on the check

plaque thus indicating that the soil was not deficient in phosphorus. The other sample was taken from a newly reclaimed area adjoining the first, and was being cultivated for the first time. The available phosphorus was not determined on this soil. The plaque test showed it to be deficient in phosphorus.

Jones' paper may be considered to be a comparison of the Truog method for determining available phosphorus and the plaque test. Experience with this method has shown that for the soils of the northern part of the United States, loams and clays should contain at least 150 p.p.m. and sands should contain 100 p.p.m. of available phosphorus for general farming purposes. Using these values as a basis for the classification of the soils reported by Jones, the Truog method shows four of the soils tested to contain sufficient phosphorus to allow them to be placed in the fertile group. The plaque test shows only one of the soils to be non-deficient.

The average phosphorus content of the soils classed as fertile by the Truog method, excluding the muck-swamp soil found to be non-deficient by the plaque test, is 180 p.p.m. The muck-swamp soil found to be non-deficient by the plaque test contained 413 p.p.m.

available phosphorus. This difference of available phosphorus content suggests that the plaque test is not as sensitive to the available phosphorus of the soil as the Truog method.

Jones prepared a series of four plaques containing 5 ml., 2.5 ml., 1 ml., and 0.1 ml. of a 3 per cent solution of K_2HPO_4, corresponding to an application of 15281 lbs., 7640 lbs., 3093 lbs., and 309 lbs., respectively, of superphosphate per acre. He found the Azotobacter colony development to be excellent, fair, poor, and very poor, respectively. Jones concluded that the optimum phosphorus content of the soil for vigorous colony formation was far in excess of that required for maximum crop returns.

Dahlberg and Brown (4) report a comparison of the Neubauer, Winogradsky (Azotobacter), and a chemical method for the determination of available phosphorus, developed in the laboratories of The Great Western Sugar Company. One hundred and one soils were tested. They varied in pH from 7.1 to 9.1 and came from the states of Colorado, Wyoming, Montana, and Nebraska. The following tabulation shows the agreement of the methods for all soils tested and the agreement for soils falling into the three Neubauer classes:

Agreement of Methods for Determining Available Phosphorus on 101 Soils

Methods compared	Agreement in all soils tested	Agreement in the Neubauer classes (deficiency)		
		Not def.	Possibly def.	Very def.
Neubauer alone		6	15	80
All methods	73	1	4	68
Neubauer and Winogradsky	84	1	5	78
Neubauer and chemical	82	5	8	69
Winogradsky and chemical	75	1	6	68

Dahlberg and Brown also report that the lack of agreement between the Winogradsky and chemical tests was due to the tendency of the former to give low results on soils of high P_2O_5 content, and the tendency of the chemical test to give high results for soils of low P_2O_5 content, in some cases. They conclude that if the Neubauer test be considered standard, the Winogradsky method is a highly satisfactory substitute on deficient soils as they agree in 78 out of 80 cases.

Field tests on the very deficient soils show that 50 per cent are so deficient in phosphorus that they cause "black heart" of sugar beets, a malnutritional condition caused by phosphorus starvation. The other 50 per cent respond markedly to phosphorus fertilization and produce one to two tons more beets per acre with an

application of 125 pounds of treble superphosphate per acre.

It is of interest to note that Dahlberg and Brown make this statement, "..... the same field which develops this disease (black heart) in sugar beets will grow very satisfactory grain crops, although grain gives a decided response to phosphorus applications."

MATERIALS AND METHODS

The Soil Samples

With the exception of two samples, all of the soils used in the investigation were taken from Series A of the Rotation and Fertilizer Experiment of the Virginia Agricultural Experiment Station. This experiment is located on Hagerstown silt loam of moderate fertility. Table I shows the fertilizer treatment of these plots from 1901 to 1931. Applications of fertilizing materials have been made annually unless otherwise noted. With the exception of the continuous corn plots, $1A_1$ and $1A_2$, all of these plots have been cropped in a four year rotation of corn, wheat and hay for two years (Mammoth clover, red top and timothy). These plots were sampled in October, 1931, just after the corn was harvested. The samples were air dried, sieved thru a 20-mesh sieve, and stored in glass jars for future use.

A sample of soil from the Horticultural propagation gardens on Stroubles Creek has been used in one of the experiments. It is a heavy soil of high fertility. A sample was also obtained in June 1932* from near Fowler, Colorado. It is a loamy soil of alkaline reaction.

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*By Mr. W. W. Allison.

Table I. Fertilizer Treatment of Plots in Series A of the Rotation Experiment.

Plot	Fertilizer Treatment*
$1A_1$	1909-1931, No treatment. Check.
$1A_2$	1909-1914, No treatment 1914-1931, 16 tons manure and 219 pounds rock phosphate
$2A_2$	1909-1931, 438 pounds of superphosphate
$3A_2$	1909-1931, 308 pounds dried blood, 438 pounds superphosphate, and 200 pounds muriate of potash
$4A_1$	1909-1931, No treatment. Check.
$4A_2$	1909-1917, No treatment 1917-1931, 200 pounds ammonium sulfate and 219 pounds rock phosphate
$5A_2$	1909-1931, 348 pounds of superphosphate, and 200 pounds muriate of potash
$6A_2$	1909-1931, 200 pounds of muriate of potash
$7A_2$	1909-1931, 308 pounds dried blood, and 200 pounds muriate of potash
$8A_2$	1909-1931, 308 pounds dried blood, and 438 pounds superphosphate
$9A_2$	1909-1931, 308 pounds of dried blood
$10A_2$	1909-1931, 16 tons manure once in 4 years before corn
$11A_1$	1909-1931, No treatment. Check.
$11A_2$	1909-1917, No treatment 1917-1931, 16 tons manure once in 4 years before corn, and 219 pounds rock phosphate
$12A_2$	1909-1931, 4 tons of manure annually
$13A_2$	1909-1931, 16 tons manure once in 4 years before corn, and 438 pounds superphosphate annually

*During the first 9 years, the fertilizer applications were one-half of that given in this table, with the exception of manure.

Available Phosphorus Determinations

The available phosphorus of both the soils and the plaques was determined by the Lamotte-Truog method. The standard equipment and reagents supplied in the kit were used. The method employs a dilute acid extraction medium and the phosphomolybdic acid colorimetric reaction of Deniges (5). Phosphorus is reported as pounds per acre.

H-ion Determinations

In the pH determinations, a Youden potentiometer was used. It employs a 1/20 molar solution of potassium acid phthalate as a buffer solution in the reference electrode vessel, and uses the quinhydrone electrode. Small test tubes were used as electrode vessels in making the determinations. Approximately 1 gm. of the soil to be tested and 5 ml. of water were placed in a test tube. The mixture was shaken thoroly and allowed to stand for 10 to 20 minutes before the quinhydrone was added and the reading taken. Experience has shown that this period of time of standing is necessary for the water and soil to come to an equilibrium, thereby giving a constant reading.

All determinations were made in duplicate. A variation of 10 m.v. has been allowed between duplicate

determinations. An average of the two readings was taken as the voltage for the sample. If the duplicate determinations varied more than 10 m.v., a third determination was made. The average of the two readings falling within the variation allowed was taken as voltage for the sample.

Source and Culture of Azotobacter Chroococcum

The strain of Azotobacter chroococcum used in these studies was obtained from Dr. Waksman. The culture has been kept in a vigorous condition by repeated transfers on nitrogen free media. All inoculations of plaques have been made from young and vigorous cultures.

Sackett's Technic for Making the Plaques

The following procedure is reported by Sackett for making the plaques (24), p. 12.

Weigh out four 50 gram portions of the soil to be tested, which has previously been air dried and sieved thru a 20-mesh sieve. Place each portion in a small class culture dish, and to each add 2.5 grams of cornstarch. If the soil is more acid than a pH of 6.8, add 5 grams of calcium carbonate to each portion. Mix thoroly while dry.

If calcium carbonate was added, add 1 ml. of a suspension of Azotobacter chroococcum*. To portion 1 add distilled water as described below; to portion 2, add 5 ml. of a 3 per cent solution of K_2SO_4; to portion 3, 5 ml. of a 6 per cent solution of Na_2HPO_4; and to portion 4, 5 ml. of a 3 per cent solution of K_2HPO_4. To portion 1 add enough distilled water, stirring continually, to bring the soil to a consistency of putty or modeling clay, noting the amount of water required. Mix thoroly, and smooth the top with a moist glass slide. Place in a moist chamber, containing a layer of wet filter paper in the bottom. To portions 2, 3, and 4, add enough distilled water to bring the total amount of liquids used up to that of portion 1 and prepare for incubation in a similar way. Incubate at 30° C. for 72 hours. At the end of this period, if the correct limiting factors have been supplied, large opalescent colonies of Azotobacter will have appeared.

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*Prepare the suspension by washing the growth from a 24 to 48 hour culture with sterile physiological salt solution, and dilute to 100 ml. with the salt solution.

Procedure Used in this Investigation for Making the Plaques

Weigh out four 50 gram portions of the soil to be tested, which has previously been air-dried and finely sieved thru a 20-mesh sieve, and place each portion in a glass tumbler. If the soil is more acid than a pH of 6.8, add 2 grams of $CaCO_3$ and inoculate by adding 1 ml. of a finely divided suspension of Azotobacter chroococcum to each portion*. Add 1 ml. of a 1-1 solution of sucrose and water, as energy material. To portion 1 add distilled water as described below; to portion 2, add 5 ml. of a 3 per cent solution of K_2SO_4; to portion 3, 5 ml. of a 6 per cent solution of Na_2HPO_4; and to portion 4, 5 ml. of a 3 per cent solution of K_2HPO_4. To portion 1 add enough distilled water to bring the soil to a consistency of putty or modeling clay, mix thoroly, transfer to a petri dish half, smooth the top with a moist spatula and place for incubation in a moist culture chamber containing a

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*Prepare the suspension by washing the growth from a young and vigorous agar slant culture with sterile distilled water into a flask of sterile water containing 1 to 2 grams of quartz sand. Shake thoroly. Dilute to a slight turbidity. The sand aids in dispersing the organisms.

small amount of water and a false bottom to support the plaques. To portions 2, 3, and 4 add enough distilled water to bring the total amount of liquids up to that used in portion 1, mix thoroly and prepare for incubation in a similar way. Incubate at room temperature (25° to 30° C.) for 72 hours. At the end of this period, if the correct limiting factors have been supplied, very characteristic Azotobacter colonies will have appeared on some of the plaques.

As most Eastern soils are more acid than a pH of 6.8, the addition of calcium carbonate and inoculation are important phases of the technic. Sackett's technic has been modified in the substitution of sucrose for starch and distilled water for physiological salt solution.

EXPERIMENTAL

The original plan of this research was to study the adaptability of the Winogradsky plaque method, as reported by Sackett, to Virginia soils. If the method was found to be satisfactory, the mineral nutrient content of soils from Grayson, Nansemond and Rockbridge Counties were to have been studied. Soil samples collected during the recent soil survey were to have been used. However, as the method was not found to be adaptable, the second part of the problem was abandoned.

The researches herein reported deal with a study of the Winogradsky method as used by Sackett and attempts to modify his technic for use on Virginia soils.

Preliminary Experiments on the Plaque Technic

Preliminary Experiments on the Plaque Technic

In a preliminary trial of the method as reported by Sackett, eight samples of soil from the series of plots described under materials and methods were plaqued for deficiency studies, and incubated at room temperature. After 72 hours incubation, no colonies had developed on the plaques. As the technic had been carefully followed in every detail except that of incubation, plaques of four soils were placed in a 30° C. incubator and plaques of the four remaining soils left in the room temperature incubator. The temperature in the latter incubator varied from 25° to 30° C.

After a further incubation period of two days, no colonies had developed on the plaques in either incubator. It was concluded that temperature of incubation had not been an important factor in preventing Azotobacter growth. Realizing that Sackett's technic had been developed for use on alkaline soils and that his procedure probably needed modification for use on acid soils, the following experiments were outlined and carried out.

Aeriation and Source of Energy Study

Sackett experienced difficulty in securing Azotobacter growth on certain soils in Colorado. These soils either lacked in the necessary flora to hydrolize starch to sugar, thus making the starch unavailable to Azotobacter, or they made such compact plaques that Azotobacter failed to grow because of lack of proper aeriation. To such soils he added 1 ml. of a 1-1 solution of sucrose or 10 grams of quartz sand to each 50 gram plaque.

To test these recommendations on a Virginia soil four series of plaques of four each were set up. When starch, sucrose, or sand were used, they were added in the following amounts per plaque: starch, 2.5 grams; sucrose, 1 ml. of a 1-1 solution; and sand, 10 grams. Calcium carbonate and nutrients were added as described under Materials and Methods. Table II shows the treatment of plaques and the response of Azotobacter. This test showed starch and sucrose to be of about equal value as energy material for Azotobacter. Starch seemed to encourage mold growth on the plaques while sucrose discouraged it. Sand decreased the vigor of Azotobacter growth with both starch and sucrose.

Table II. The Effect of Starch, Sucrose and Sand on Azotobacter Growth.

Series	Plaque treatment	Azotobacter growth with various nutrients added			
		None	K_2SO_4	Na_2HPO_4	K_2HPO_4
A	Starch	*---	---	A few well developed colonies. Very moldy	A few well developed colonies. Very moldy
B	Sucrose	---	---	Same as (A) No mold	Same as (A) No mold
C	Sand and starch	---	---	Less vigorous than (A) No mold	Less vigorous than (A) No mold
D	Sand and sucrose	---	---	Poor growth No mold	Poor growth No mold

*No growth.

The Effect of Sucrose, Dipotassium Phosphate and Varying Amounts of Calcium Carbonate on the pH of the Plaques

The work of Gainey (10) definitely shows that the reaction of the culture medium determines to a marked degree the growth and longevity of Azotobacter. The soils used in this study are slightly acid. It was felt that a thoro study of the effect of the materials used in making the plaques upon their pH should be made.

Nine plaques of a soil having an initial pH of 6.2 were set up in the regular way except for the treatments shown in Table III.

Table III. The Effect of the Materials used in Making the Plaques on their pH.

Plaque No.	Plaque treatment	pH at different determinations		
		24 hrs.	48 hrs.	72 hrs.
1	Check, water only	5.8	5.4	6.2
2	Water and sucrose	5.5	5.3	6.2
3	Water and K_2HPO_4	5.9	5.6	6.4
4	Water, sucrose and K_2HPO_4	5.9	5.6	6.4
5	Treatment (4) and 0.25 gm. $CaCO_3$	6.6	6.5	7.2
6	Treatment (4) and 0.50 gm. $CaCO_3$	6.6	6.6	7.3
7	Treatment (4) and 1 gm. $CaCO_3$	6.7	6.4	7.2
8	Treatment (4) and 2 gm. $CaCO_3$	6.7	6.5	7.0
9	Treatment (4) and 5 gm. $CaCO_3$	6.7	6.5	6.9

Determinations were made when the plaques were 24, 48, and 72 hours old. This table shows that the pH of the plaques has a tendency to decrease on the second day and increase on the third day of incubation. The addition of sucrose to the plaques has a tendency to decrease the pH, while dipotassium phosphate tends to increase it. Calcium carbonate, in a concentration as low as 0.5 per cent brought this soil to approximate neutrality at the end of three days.

Effect of Varying Amounts of Calcium Carbonate on Azotobacter Growth

Sackett recommended the addition of 5 grams of $CaCO_3$ to each 50 gram portion of the soil when it had an initial pH of less than 6.8. This recommendation was based on results secured by Gainey (10), who found this quantity of calcium carbonate necessary to correct the acidity of a soil having an initial pH of 4.0. As the soils used in this test were not that acid, a test was designed to study the effect of varying amounts of calcium carbonate on Azotobacter growth. Table IV shows the amount of carbonate added and the Azotobacter growth.

Table IV. The Effect of Calcium Carbonate on Azotobacter Growth.

Gms. $CaCO_3$ added	Azotobacter growth at the end of 72 hrs. incubation
0.0	None
2.0	Very vigorous
5.0	Vigorous

This test clearly shows the necessity of the addition of calcium carbonate to the plaques for Azotobacter growth on this soil. Two grams gave slightly more vigorous growth than 5 grams.

Sodium Carbonate as a Substitute for Calcium Carbonate

It is generally accepted that the available phosphates in a soil are reverted to the tricalcium form in the presence of large amounts of calcium carbonate. This reversion could occur in the plaques, and thereby change the phosphorus of a soil to a form that would be unavailable to Azotobacter. If some other material could be substituted for calcium carbonate in making the plaques, this possible source of error might be eliminated.

Sodium carbonate was found by Gainey (10) to be a satisfactory soil amendment for Azotobacter growth. This material was added to the plaques in varying amounts and the effect on Azotobacter growth noted. Soil $1A_1$ was used. To 50 gram plaques of soil 0.25, 0.5, 0.6, 0.7, 0.8, 0.9, and 1.0 grams of sodium carbonate were added. A plaque containing 2 grams of calcium carbonate was used as the control. None of the sodium carbonate plaques produced colonies, while the calcium carbonate plaque produced very good growth. It was concluded that calcium carbonate was probably the most satisfactory amendment for use in the plaques.

A Comparison of Physiological Salt Solution, Distilled Water and Tap Water as Liquids for Azotobacter Suspensions

The growth from three vigorous cultures of Azotobacter was washed from the slants with sterile physiological salt solution (.85 per cent), distilled water, and tap water. The suspensions were shaken to separate the organisms and were diluted to produce approximately the same turbidity. These suspensions were streaked on plates of Lipman's mannitol nitrogen-free agar. All three suspensions produced growth of approximately equal vigor at the end of the 72 hour incubation period.

A Test of Strains and Species of Azotobacter for Vigor of Growth on the Plaques

To secure organisms for this study, requests were sent to four Experiment Station laboratories for transfers of the cultures in stock. Eight cultures were received, including 3 species. The following tabulation shows the strain or species, investigator, and the laboratory from which they came.

Species	Investigator	Experiment Station Laboratory
Azotobacter chroococcum (St. 73)	Sackett	Colorado
Azotobacter chroococcum (St. 62)	Sackett	Colorado
Azotobacter chroococcum (St. 58)	Sackett	Colorado
Azotobacter chroococcum	Walker	Iowa
Azotobacter chroococcum	Wilson	New York
Azotobacter chroococcum	Waksman	New Jersey
Azotobacter Beijerinckii	Waksman	New Jersey
Azotobacter vinelandii Lipman	Waksman	New Jersey

A culture of Azotobacter isolated from soil taken from the edge of a compost pile behind the Plant Pathology green house was also included in the study.

Large plaques were made in soup plates, using 200 grams of soil. Nutrients were added in the regular way and in corresponding amounts. Suspensions of the organisms were made in physiological salt solution (.85 per cent) and diluted to approximately the same turbidity. The suspensions were streaked along parallel lines on the plaques with sterile cotton swabs.

Only the Na_2HPO_4 and the K_2HPO_4 plaques developed colony growth. All of the cultures gave very good growth on these plaques. However, strain 73, Sackett, was less vigorous than the other cultures of A. chroococcum.

A. Beijerinckii and vinelandii Lipman produced slightly less vigorous growth than the strains of A. chroococcum. The organism from the local soil was less vigorous than the strains of A. chroococcum. It produced growth of the same vigor as Beijerinckii or vinelandii Lipman.

A Comparison of the Streaking and Mixing Methods of Inoculating the Plaques

Sackett recommended that 1 ml. of a suspension of Azotobacter be mixed with the soil along with the nutrient solutions, when inoculation was necessary. If the soil and inoculum were not thoroly mixed, all of the organisms might be covered with the soil and be unable to develop colonies because of lack of aeriation.

By streaking the organism on a localized area of the surface of the plaques with a sterile cotton swab this possibility would be eliminated. Table V. shows a comparison of the two methods of inoculation on duplicate plaques of 5 soils. This experiment indicated that the streaking or mixing method were equally reliable for inoculating the plaques.

Table V. A Comparison of the Streaking and the Mixing Methods of Inoculation.

Soil	Nutrients added and methods of inoculation							
	None		K_2SO_4		Na_2HPO_4		K_2HPO_4	
	Str.	Mix.	Str.	Mix.	Str.	Mix.	Str.	Mix.
	1*				2*			
1	---	---	---	---	X	X	X	X
2	---	---	---	---	---	---	X	X
3	---	---	---	---	---	---	X	X
4	---	---	---	---	X	X	X	X
5	---	---	---	---	---	---	X	X

Notes: 1 No growth; 2* Growth.

The Effect of the Nutrient Solutions on the pH of the Plaques

Table VI shows the effect of the nutrient solutions used in making the plaques upon the H-ion concentration at the end of 72 hours incubation. The plaques were prepared in the usual way. Two grams of calcium carbonate and 1 ml. of inoculum were added to soil $13A_2$; neither were added to the Colorado soil.

Table VI. The Effect of Plaque Conditions and the Nutrient Solutions on the pH of the Plaques.

Soil	Original pH	pH of nutrient plaques			
		None	K_2SO_4	Na_2HPO_4	K_2HPO_4
Colorado	8.4	6.9	7.0	7.5	7.0
$13A_2$	6.6	7.8	6.9	7.5	7.1

The decrease in pH of the no nutrient plaque of the Colorado soil over the original pH is very marked. All of the plaques to which the nutrient solutions were added, except the K_2SO_4 plaque of soil $13A_2$, gave significant increases in pH. The Na_2HPO_4 plaques were markedly higher in pH.

Effect of Varying Quantities of K_2HPO_4 on Azotobacter Growth

To study the effect of varying quantities of phosphate on the growth of Azotobacter, eight plaques of soil $1A_1$ were prepared in the usual way. The 3 per cent solution of K_2HPO_4 was added in varying amounts. Table VII shows the amounts of phosphate solution added and the response of Azotobacter on the plaques.

Table VII. The Effect of Varying Amounts of Dipotassium Phosphate on Azotobacter Growth.

Ml. 3 per cent K_2HPO_4	Growth 48 hrs.	Growth 72 hrs.
0.0	None	None
0.5	Very slight	Very slight
1.0	Slight	Slight
2.0	Poor	Poor
3.0	Fair	Fair - Good
4.0	Good	Good
5.0	Very good	Very good
10.0	Slight	Fair

On this soil, which is low in available phosphorus 10 to 25 pounds per acre (LaMotte-Truog), the Azotobacter growth is directly proportional to the amount of dipotassium phosphate added. Five ml. of the phosphate solution produced very good growth while 10 ml. depressed growth.

Azotobacter Growth on Plaques of Varying Moisture Content

During these studies, it was observed that plaques in which the moist soil or mud was soft, did not produce vigorous Azotobacter growth. To test this observation, 5 plaques were prepared, using a soil that previously gave good growth with 18 ml. of liquids added per plaque. This included the nutrient solution, sucrose, inoculum and distilled water.

Table VIII shows the total liquids added to each plaque, the character of the moist soil or mud, and the Azotobacter growth at the end of 72 hours incubation. The plaques that were stiff, or had the plasticity of putty or modeling clay, gave the most vigorous growth. The detrimental effect of making the plaques too moist is clearly shown.

Table VIII. The Effect of Varying the Moisture Content of the Plaques on Azotobacter Growth.

Ml. of liquids added	Character of the moist soil	Azotobacter growth
16	Very stiff	Good
17	Plasticity of putty or modeling clay	Good
18	Plasticity of putty or modeling clay	Good
19	Soft	Poor
20	Very soft	Very poor

The Effect of Calcium Nitrate on Azotobacter Growth

It is generally accepted that small amounts of nitrates are utilized by Azotobacter. In an attempt to supply the nitrogen necessary for Azotobacter growth, calcium nitrate was added to 5 plaques of soil in quantities to produce a concentration of 10, 20, 40, and 100 p.p.m. The Azotobacter growth was decreasingly vigorous as the concentration of nitrates increased. The check plaque, containing no nitrates, produced very good growth while the plaque containing 100 p.p.m. produced feeble growth at the end of 72 hours incubation. There was a tendency for the plaques containing the higher concentrations to start growth about 24 hours later than the check.

Summary of Preliminary Experiments

Observations during these studies have confirmed Sackett's suggestions concerning the moisture relationships of the atmosphere of the incubation chamber and of the moist soil or mud. A low moisture content of the soil and a high moisture content of the air in the culture chamber are essential to good Azotobacter growth. Sand tends to decrease the vigor of growth, when used in 20 per cent concentration, with starch or sucrose as an energy material. These two carbohydrates are of about equal value as sources of energy for use in the plaques. Sucrose has been used in these studies because of its convenience.

Sackett (24), p. 11, states that Azotobacter has been grown in the presence of 33 per cent calcium carbonate without any deleterious effects. These studies have shown that on these soils a concentration of 4 per cent allows slightly more vigorous growth than a concentration of 10 per cent. Sodium carbonate was not found to be a satisfactory substitute for calcium carbonate.

Several factors seem to be constantly active in influencing pH in the plaques. Sucrose tends to decrease it. Dipotassium phosphate tends to increase it. When

both materials are present, each seems to have a counteracting influence upon the other. An acidity cycle seems to occur in the plaques. During the first and second days of incubation, the pH is decreasing or remains constant. At the end of the third day, it has risen and usually approaches neutrality, when calcium carbonate has been used in the plaques.

This acidity cycle is probably associated with the hydrolysis of the sucrose and its conversion into organic acids. The acids accumulate in the plaque. When it becomes sufficiently acid, the calcium carbonate goes into solution, neutralizes the acids, and the pH approaches neutrality.

It has been observed that Azotobacter grows most rapidly when the plaques are 48 to 72 hours old. Gas "puffs" on the plaques occur very frequently during this period. Possibly the carbon in the sucrose cannot be utilized by the soil organisms until it has been converted into a calcium ester of an organic acid.

Physiological salt solution, distilled water and tap water have been found to be of equal value as liquids for suspensions of Azotobacter. Sackett (24) used physiological salt; Jones (12) used distilled water. Observation has shown that a faintly turbid suspension

produces distinct colonies; while a suspension, of such turbidity that it is not transparent, produces a gelatinous film over the surface of the plaques. Azotobacter chroococcum, A. Beijerinckii, or A. vinelandii Lipman can be used satisfactorily for inoculating the plaques. Streaking the inoculum on the surface of the plaques or mixing it with the soil are equally reliable as methods of inoculation.

Disodium phosphate has a tendency to increase the pH of the plaques over that of either the potassium sulfate or dipotassium phosphate plaque.

The concentration of phosphorus necessary for vigorous colony formation, on the surface of the plaques, is much higher than that necessary for the growth of an average field crop. This concentration is necessary because very likely the normal mode of occurrence of Azotobacter in the soil is a single cell or in small groups of cells clinging to the colloidal covering of the soil particles. If large colonies are to be produced on the surface of the plaques in 72 hours, a readily available supply of phosphorus must be present, as this element is one of the first limiting factors in Azotobacter growth.

Results Secured with the Winogradsky Method in Comparison with Corn Yields and Available Phosphorus of Plot Soils

Results Secured with the Winogradsky Method in Comparison with Corn Yields and Available Phosphorus of Plot Soils

As a trial of the Winogradsky method on field soils 16 samples from plots of series A of the Rotation and Fertilizer Experiment of the Virginia Agricultural Experiment Station, Blacksburg, were used. The fertilizer treatment and cropping system employed on these plots is given in Table I, page 18.

The fertilizer treatment has varied from that of the unfertilized checks to that of a plot receiving liberal applications of nitrogen, phosphorus and potassium. The corn yield in 1931 varied from 10 to 68 bushels per acre. The available phosphorus varied from 10 to 75 pounds per acre in the samples used in this study. The wide variation in the fertilizer treatment, corn yield, and available phosphorus of these plots make them adaptable for a study of the Winogradsky method in accordance with the procedure given on page 22.

The response of these soils to the Winogradsky method is shown in Table IX. None of the soils produced colonies on the unfertilized plaque or on the plaque to which potassium sulfate had been added. Growth developed

on seven of the plaques to which disodium phosphate had been added and all sixteen produced growth when dipotassium phosphate was added. Using the system of classification of deficiencies suggested by Maxson, page 10, all of these soils were very deficient, according to their response to the Winogradsky plaque test.

The corn yields and available phosphorus of these plots do not agree with the results secured with the Winogradsky method. Plot $1A_1$ received no fertilizer from 1909 to 1931 and yielded at the rate of 17 bushels of corn in 1931. Over the same period, plot $3A_2$ received liberal quantities of fertilizer for good corn production as indicated by its yield of 68 bushels. Both plots responded in a similar way to the plaque test.

All of the soils that produced colonies on the disodium phosphate plaque yielded at the rate of 50 bushels or more per acre in 1931. This indicates that this group of soils are relatively fertile, but the phosphorus or potassium fertilization is not consistently associated with this response to the plaque test.

Five of these soils contained 75 pounds of phosphorus per acre. This quantity is considered sufficient for average cropping needs on clays and loams. None of the five produced colonies on the unfertilized plaque.

Table IX. A Comparison of Fertilizer Treatment, Available Phosphorus, Corn Yields and the Plaque Test.

Soil	pH	Fertilizer treatment	Lbs. Phos per A.	Corn yield bu. per A., '31	Nutrients added: None	K_2SO_4	Na_2HPO_4	K_2HPO_4
$1A_1$	5.6	Check	25	17.00	1* ---	---	---	2* XX
$1A_2$	6.4	'17-'31. Manure Rock phosphate	75	43.15	---	---	---	XX
$2A_2$	5.7	Superphosphate	50	50.04	---	---	3* X	XX
$3A_2$	5.5	Dried blood Superphosphate Mur. of potash	50	68.86	---	---	---	XX
$4A_1$	6.2	Check	10	49.86	---	---	---	XX
$4A_2$	5.4	Ammonium sulfate Rock phosphate	75	58.00	---	---	---	XX
$5A_2$ F	5.8	Superphosphate Mur. of potash	50	61.71	---	---	X	XX
$6A_2$	7.0	Mur. of potash	10	51.43	---	---	4* X	XX
$7A_2$	6.4	Dried blood Mur. of potash	10	54.00	---	---	---	XX
$8A_2$	6.3	Dried blood Superphosphate	75	55.29	---	---	4* X	XX
$9A_2$	6.1	Dried blood	10	31.14	---	---	---	XX
$10A_2$	6.7	Manure (Rotat.)	50	65.71	---	---	X	XX
$11A_1$	6.1	Check	10	34.86	---	---	---	XX
$11A_2$	6.8	'17-31. Manure Rock phosphate	75	64.86	---	---	---	XX
$12A_2$	6.9	Manure (ann.)	25	63.73	---	---	X	XX
$13A_2$	6.6	Manure Superphosphate	75	67.13	---	---	X	XX

Notes: 1 No growth; 2* Vigorous; 3* Poor growth; 4* Very poor growth.

It is evident that the Winogradsky and the LaMotte-Truog methods do not agree on the phosphorus content of these soils. The LaMotte-Truog method is generally considered to be a satisfactory indicator of the phosphorus content of soils. This indicates that the Winogradsky method is not sensitive enough to reflect the presence of sufficient nutrients for the production of a good corn crop.

Miscellaneous Studies on the Plague Test

A Comparison of Azotobacter Growth on Plaques of a Colorado and a Virginia Soil

Observation of Azotobacter growth on plaques of the Virginia soils had shown that it was probably not as vigorous as that shown and described by Sackett (24) for Colorado soils. To test this observation, plaques were set up in the regular way using soil $13A_2$ and the Colorado soil.

The Virginia soil was 6.6 in pH and contained 75 pounds of available phosphorus. The Colorado soil was 8.2 in pH and contained 50 pounds of available phosphorus per acre. Calcium carbonate and inoculum were added to the former soil; neither were added to the latter. Plates II and III show the Azotobacter growth on these soils. The Colorado soil produced large opalescent colonies that resembled those described by Sackett; soil $13A_2$ produced small, feeble colonies.

The vigorous growth on the Colorado soil could have been due to the presence of a particularly vigorous strain or species of Azotobacter. To test this possibility, two plaques of soil $13A_2$ were inoculated with a suspension of Azotobacter taken directly from the plaques of Colorado soil. This was set up in sterile vessels,

so as to avoid introducing other Azotobacter. A plaque of the same soil inoculated with the stock culture of Azotobacter chroococcum was used as the control. At the end of 72 hours incubation, all plaques had produced colonies of about equal vigor. The organism taken directly from a plaque of Colorado soil produced slightly more pigment than the stock culture.

The Effect of Varying Amounts of Calcium Carbonate on the Availability of the Native Phosphorus of a Soil

Calcium carbonate was used in the plaque tests previously reported on the plot soils at a concentration of 4 per cent. It is generally accepted that a reversion of available phosphates occurs in the presence of large amounts of $CaCO_3$. A reversion of the phosphates present in these soils could have occurred, thereby making the available phosphorus content of all soils low, as the results secured with the Winogradsky method suggest.

To find the amount of $CaCO_3$ necessary to revert the available phosphorus of a relatively fertile soil, the following test was outlined and carried out. Soil from the Horticultural propagation garden was used. It had a pH of 7.4 and contained 75 pounds of available phosphorus

per acre. Five 50 gram plaques were made. The $CaCO_3$ was varied from 0.0 to 5.0 grams per plaque. Sucrose was added to each plaque.

Determinations of available phosphorus were made with the standard reagents and equipment of the LaMotte-Truog kit. Care was taken to pack the soil into the measuring ladle to about the same firmness for each determination. The available phosphorus is reported in numbers corresponding to the first three shades of blue on the colorimetric chart supplied with the kit. The lowest amount of phosphorus observed is indicated by the figure "1"; the intermediate amount observed is represented by the figure "2"; the highest by the figure "3".

Table X shows the relative amounts of available phosphorus on the variously treated plaques at different intervals over a period of 8 days.

Table X. The Effect of Calcium Carbonate on the Availability of the Native Phosphorus of a Soil.

Gms. $CaCO_3$	Relative amounts of available phosphorus at different ages of the plaques				
	24 hrs.	48 hrs.	72 hrs.	120 hrs.	192 hrs.
0.0	1	1	2	3	3
0.1	3	2	2	3	3
0.5	2	1	2	3	3
1.0	2	1	2	2	3
2.0	2	1	2	2	3
5.0	1	1	1	1	1

This table shows that a 5 gram application of $CaCO_3$ changes the available phosphorus present in this soil to a form that is insoluble in the extracting solution used in the LaMotte-Truog method. Smaller applications did not influence the solubility of the phosphorus of this soil in the reagents used. This suggests that little, if any, reversion occurred in the plaques of the plot soils, Table IX, as 2 grams have been used thruout the work.

The Effect of Varying Amounts of Phosphate on the Available Phosphorus of the Plaques

To determine the effect of the 2 grams of $CaCO_3$ used in making the plaques on the phosphate added, the following test was carried out. Varying amounts of a 3 per cent K_2HPO_4 solution were added to plaques of soil $1A_1$. This soil contains 10 to 25 pounds of available phosphorus per acre. Sucrose and $CaCO_3$ were added to the plaques in the regular amounts. Determinations of the available phosphorus were made as previously described.

Table XI shows the relative amounts of available phosphorus in the plaques at intervals over a period of three days.

Table XI. The Effect of Two Grams of Calcium Carbonate on the Available Phosphorus of Plaques Containing Varying Amounts of Phosphate.

Ml. of 3 per cent K_2HPO_4	Relative amounts of available phosphorus at different ages of the plaques				Azotobacter Growth
	2 hrs.	24 hrs.	48 hrs.	72 hrs.	72 hrs.
0.0	1	1	1	2	None
0.5	1	1	1	2	None
1.0	4	3	4	4	Slight
2.0	5	4	4	4	Poor
3.0	7	4	5	4	Fair
4.0	7	6	5	4	Good
5.0	7	7	5	5	Good
10.0	7	7	6	6	Poor

This test shows that a small part of the phosphate added to a soil gradually changed to a form that was insoluble in the extracting solution used by the LaMotte-Truog method. The insoluble portion could have been assimulated by Azotobacter or reverted. These data suggest that reversion occurred, in that the plaque to which 0.5 ml. of the phosphate solution was added contained approximately the same amount of available phosphorus as the check plaque and did not produce colony growth.

A Study of the Adaptability of Algae to the Plaque Test

The Azotobacter group of organisms are not chlorophyll bearing and do not synthesize energy materials. As a result, carbohydrate must be added to allow the organism to develop on the plaques. In the hydrolysis and subsequent utilization of the carbohydrate by Azotobacter, organic acids are produced that tend to increase the acidity of the soil. This condition plays a part in making the addition of $CaCO_3$ necessary to keep the reaction within an optimum range for Azotobacter growth. The combined action of the organic acids and the $CaCO_3$ will probably change the normal biological, chemical and physical equilibria of the soil, thereby producing a condition entirely different from those existing under field conditions.

The growth of algae on the plaques as a substitute for Azotobacter would eliminate the addition of both carbohydrate and $CaCO_3$, since the former synthesizes its energy material by photosynthesis.

A test was devised to determine the best method of treatment of the plaques to allow the best visibility of the algae growth. Four plaques were made in petri dish halves using 50 grams of soil, 5 ml. of a 3 per cent

solution of K_2HPO_4 and enough 0.1 per cent $Ca(NO_3)_2$ solution to bring them to a putty-like consistency. They were divided into 4 groups of 2 each, treated as indicated in Table XII, and inoculated by dropping a suspension of a mixed culture of algae over the top of the finished plaques. The dishes were covered with regular petri dish lids and incubated in a northern window, where they could receive light. Table XII shows plaque treatment and algae response.

Table XII. The Effect of Surface Treatment on the Growth and Visibility of Algae on the Plaques.

Plaque Treatment	Algae Growth
Filter paper pressed firmly over the soil	Poor growth
Dusted with kaolin, and left rough	Excellent growth, and visibility
Dusted with kaolin, and smoothed with a moist spatula	Fair growth
Left bare	Poor growth Poor visibility

This study shows that dusting the surface of the plaques and leaving it rough for the alga to grow on, gives the most vigorous growth and best visibility of the growth.

In order to test the adaptability of algae to the plaque test, 2 duplicate series of 4 plaques each of soil $1A_1$ were set up. The plaques were made in the regular way, except that the sucrose and $CaCO_3$ were omitted, and a 0.1 per cent solution of calcium nitrate was substituted for the distilled water. They were dusted with kaolin and inoculated, and incubated as previously described. Table XIII shows the treatment and response of the algae.

Table XIII. The Use of Algae on the Plaques as a Substitute for Azotobacter.

Nutrients Added	Algae Growth at the end of 7 days
None	Vigorous Growth
K_2SO_4	Vigorous Growth
Na_2HPO_4	Vigorous Growth
K_2HPO_4	Vigorous Growth

After 7 days incubation, all of the plaques showed growth of about equal vigor. It is evident that algae is not a suitable organism to grow on the plaques as a substitute for Azotobacter since it grew equally as well on the plaques to which no additional nutrients had been applied as it did on the plaque receiving potassium, phosphorus, or the two in combination.

Discussion

The first part of this investigation was concerned with a study of the plaque technic in which the materials used in making the plaques were varied in an attempt to find optimum conditions for Azotobacter growth. These experiments were discussed and summarized on pages 38 to 40. After the completion of the experiments therein described, it was felt that the technic had been modified for use on Virginia soils and that the author was sufficiently familiar with it for a test of the adaptability of the Winogradsky test to be made.

The corn yields and available phosphorus of the soils from the 16 plots of the experiment used in this study show that their fertility varies to a marked degree. The response of these soils to the Winogradsky plaque test does not reflect this variation. Two possible causes for this lack of sensitivity suggested themselves. The available phosphorus could have been reverted in the plaques, as calcium carbonate had been used at a concentration of 4 per cent; or Azotobacter could be unable to produce colonies on a soil containing sufficient phosphorus for the production of good corn yields.

Stolenska (29) reports the time limit for reversion of phosphorus in the soil as 50 to 60 days; Ergorov (7) found it to occur in 24 days; Shapiro (26) claims it occurred in 10 days. Ergorov further states that rapidity of reversion varies in different soils. If reversion was a factor in causing the lack of sensitivity of the plaque test on these soils, it must have occurred much more rapidly than it has previously been reported as taking place.

Table X indicates that some reversion had occurred at the end of 24 hours in plaques containing 10 per cent calcium carbonate. The concentration of 4 per cent carbonate used in these tests had little effect, if any, on the available phosphorus of a soil containing 75 pounds per acre. A small amount of reversion probably occurs when the phosphate solution is added to the plaques, Table XI. However, the indications are that reversion has not influenced the results secured with the plaque test on these soils, in that calcium carbonate at a concentration of 4 per cent caused no perceptable reversion of the native phosphorus of a soil containing 75 pounds of available phosphorus per acre.

In America the Winogradsky plaque test has been adopted and used most extensively, as far as the author

has been able to determine, in connection with the sugar beet industry. The Neubauer tables (6) show that the phosphorus and potassium content of the soil, for the production of high yields of sugar beets, to be 10 times that necessary for high yields of wheat and oats; 5 times that necessary for red clover hay; and 1.25 times that necessary for potatoes.

The work of Jones (12) and tests carried out in these studies show the concentration of phosphorus necessary for the production of vigorous Azotobacter growth on the plaques to be far in excess of that required for the common field crops. The concentration of phosphorus in soils that will produce high yields of corn as shown by Table IX, and the concentration necessary for the small grains as shown by the Neubauer tables, is low when compared with the concentration necessary for high yields of sugar beets.

The statement of Dahlberg and Brown (4), cited on page 16, is of especial interest in this connection. They state that soils so deficient in phosphorus that they cause a malnutritional condition, due to phosphorus starvation (black heart), produce satisfactory yields of the small grains, altho grain gives a decided response to phosphorus fertilization. This further suggests that

a soil may be very deficient for the production of sugar beets and moderately deficient or not deficient for small grains and corn.

The data herein reported show that Azotobacter will not produce colonies on the distilled water plaques of soils containing 50 to 75 pounds of available phosphorus per acre. This response indicates that these soils are very deficient. They are probably very deficient for the production of sugar beets, but the corn yields for 1931 show that they produced a satisfactory crop. The relationship between phosphorus fertilization and the available phosphorus of the soils indicates that the phosphorus applied in the spring of 1931 was not exhausted. The presence of these phosphate residues was not reflected by the plaque test.

Another factor influencing the adaptability of the Winogradsky plaque test to Eastern soils is their acid condition. In the application of the method, these soils must be corrected with calcium carbonate and inoculated. If seemingly reliable results were secured, they would be of questionable value. Results secured on the alkaline or very slightly acid plaques might not be adaptable to the acid soils of the field. Maxson (15) states that he doubts the value of the Winogradsky method

for use on acid soils. Smith (28), after preliminary work with the method, felt that it was of little value for use on eastern soils.

Plates II and III show the vigor of Azotobacter growth on soils from Colorado and Virginia. The Colorado soil, having an initial pH of 8.4, produced vigorous growth without the addition of calcium carbonate and inoculum. The soil from Virginia, having an initial pH of 6.6, produced less vigorous growth than the Colorado soil after the acidity had been corrected and inoculum added.

It is the author's opinion that the Winogradsky plaque test is primarily adapted for use on soils of alkaline reaction that are to be cropped to sugar beets and other plants having high mineral nutrient requirements. The principle may be adopted for use on acid soils if some acid tolerant organism could be substituted for Azotobacter. A mixed culture of algae has been studied. It was found to lack in the necessary sensitivity to mineral nutrient concentration for use on the plaques.

Summary and Conclusions

The data herein reported show that the Winogradsky plaque test does not reflect the relative fertility of soils that vary widely in crop productiveness and available phosphorus. Soil from a plot producing 17 bushels of corn and soil from a plot producing 68 bushels of corn in 1931 responded in a similar way to the plaque test. Soils containing sufficient available phosphorus for average cropping needs and soils which do not contain sufficient available phosphorus for average cropping needs, as shown by the LaMotte-Truog method, also responded similarly. This indicates that the Winogradsky plaque test is of little value for the study of the mineral nutrient content of soils, slightly acid, on which crops are to be grown that have a mineral nutrient requirement as low as that of corn.

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EXPLANATION OF PLATES

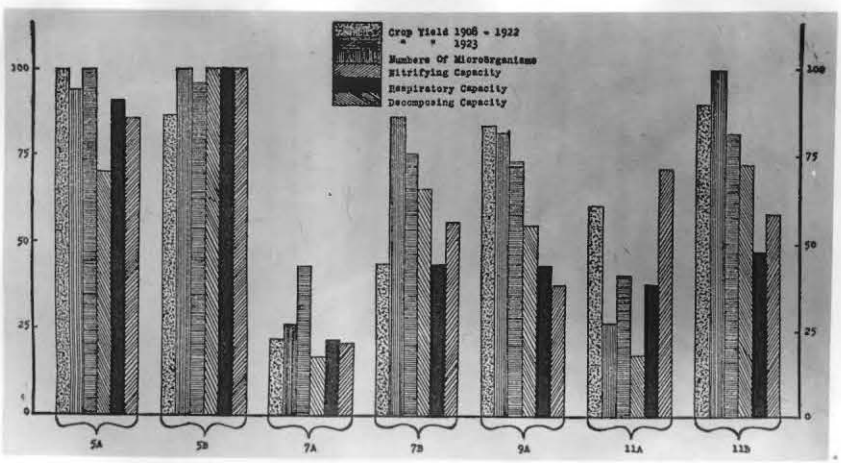
Plate I. The correlation between microbiological activities and crop yields on a series of variously fertilized plots. After Waksman and Starkey (35).

Plate II. Azotobacter growth on plaques of soil from Colorado.
Nutrients added, left to right: 1, none. 2, K_2SO_4. 3, Na_2HPO_4. 4, K_2HPO_4.

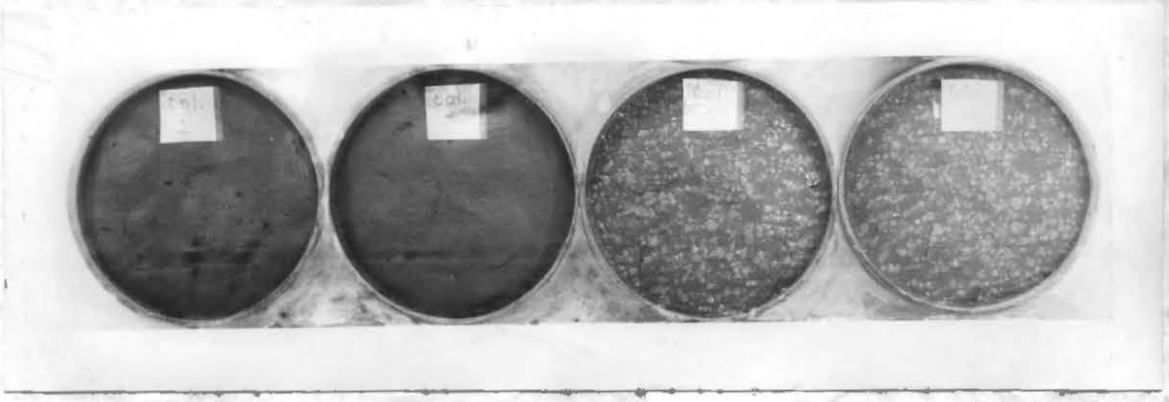
Plate III. Azotobacter growth on plaques of soil from Virginia.
Nutrients added, left to right: 1, none. 2, K_2SO_4. 3, Na_2HPO_4. 4, K_2HPO_4. Plaque 1 shows slight mold growth.

Plate IV. Azotobacter growth on plaques of the Colorado and Virginia soils. (Upper row, Virginia soil; lower row, Colorado soil).
Nutrients added, left to right: 1, none. 2, K_2SO_4. 3, Na_2HPO_4. 4, K_2HPO_4.

P L A T E I


Crop Yield 1908 - 1922
" " 1923
Numbers Of Microorganisms
Nitrifying Capacity
Respiratory Capacity
Decomposing Capacity
100
75
50
25
0
5A
5B
7A
7B
9A
11A
11B

PLATES II AND III



II

III

P L A T E IV