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COMMISSION III DE L'ASSOCIATION INTERNATIONAL DE LA SCIENCE DU SOL

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JACOB GOODALE LIPMAN

THIS volume of Proceedings of the Third Commission of the International Society of Soil Science is dedicated to the memory of Dr. Jacob Goodale Lipman, formerly Director of the New Jersey Agricultural Experiment Station and Dean of the College of Agriculture of Rutgers University. Dr. Lipman's sudden death, on April 19, removed from the field of soil science one of its most brilliant investigators. Only a few days before his death, he spent considerable time planning for this conference, which was made possible largely through his efforts.

Dr. Lipman was very active in the organization of the International Society of Soil Science and especially of the Third Commission on Soil Microbiology and Soil Biochemistry. In attendance at the Third International Agropedological Conference held in Prague, Czechoslovakia, in 1922, he was elected one of the two Presidents of the Third Commission of the Conference. In 1924, he headed the American delegation to the Fourth International Soil Science Conference held at Rome, Italy, where he acted also as one of the presiding officers of the Third Commission. At that conference, the International Society of Soil Science was organized and Dr. Lipman was elected its first President. The first Congress of the new Society was held in Washington, D. C., in 1927. For the outstanding success of the Congress and of the great excursion throughout the United States and Canada following the Congress, Dr. Lipman was largely responsible. He subsequently attended the meetings of the Third Commission of the Society, held in Stockholm, in 1929, and of the Third Congress of the Society, held at Oxford, England, in 1935. Dr. Lipman's life and work are thus intimately connected with the progress and development of the International Society of Soil Science. In dedicating this volume of Proceedings to him, we are honoring a great soil scientist.

PREFACE

THE plan to hold the Third International Congress for Microbiology in New York City during the first week in September suggested the desirability of inviting the Third Commission of the International Society of Soil Science to hold its preliminary meeting, preceding the 1940 Congress, in New Brunswick, which is in close proximity to New York City. The program of the Third Commission has been arranged in close cooperation with Subsection I on Soil Microbiology of Section VIII on Agricultural and Industrial Microbiology of the Microbiological Congress.

The following Organizations and Institutions are sponsors of these meetings:

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Opportunity is taken here to express the sincere thanks of the organizing committee to those who have contributed funds to make the publication of these Proceedings possible:

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SELMAN A. WAKSMAN

New Brunswick, N. J., U. S. A.
June, 1939

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A TRIBUTE

DR. D. J. HISSINK

Acting President and Honorary General Secretary of the International Society of Soil Science, Groningen, Holland

IN APRIL 1922, on the occasion of the Third International Conference of Soil Science at Prague, I first made Lipman's acquaintance. This Conference was the first at which workers in the same branch of science and from all parts of the world forgathered after the war; the Third International Conference of Soil Science was the first post-war gathering which was really international.

The United States had delegated to Prague two of its greatest soil scientists, Lipman and Marbut. Obviously, both of them put their stamps on the discussions in Prague and on the resolutions passed there, and thus on the course of international cooperation in the field of soil science. There can be no doubt that it was the great influence of these two which led at the time to the idea of holding a meeting of soil scientists in the United States as soon as possible.

In May 1924, at the Fourth International Conference of Soil Science at Rome, both of these colleagues were again present. As is well known, at the end of this Conference, on May 19, 1924, the International Society of Soil Science was founded. It was further resolved to hold the First International Congress of Soil Science at Washington, D. C., U. S. A., in 1927. This latter resolution entailed the choice of the first president of the Society from among the American members. It was a difficult decision that the meeting then had to make. It was a question of either Lipman or Marbut, both eminent soil scientists, both of an absolutely honorable and upright character, both enjoying the regard and affection of their colleagues. The choice, I repeat, was a difficult one; it eventually fell upon Lipman. The decisive factor was doubtless Lipman's great talent for organization. Our young Society had to cut a good figure at its first Congress in 1927, both from the scientific and from the social point of view. On this first step much depended, and in Rome we considered that this could best be achieved under Lipman's leadership.

From 1924 to 1927 Lipman prepared for the First International Congress of Soil Science. He had the cooperation of all his colleagues and of all the public bodies concerned, both in the United States and in Canada; these he united into one large Organizing Committee. He found Marbut ready to undertake the management of the great excursion through the United States and Canada. In all this work he placed himself as little as possible in the foreground; the Chairman and the Secretary of the Organizing Committee were Schreiner and McCall, and, as already mentioned, Marbut was entrusted with the management of the excursion. Lipman and these formed "The Big Four" of 1927. Lipman's great organizing talent was best shown by the fact that all, without exception, gladly worked together under his leadership.

It is obvious that in the organization of the Congress and the excursion not only the scientific and the technical, but also the financial aspect played a great part. Many colleagues outside America, who were anxious to take part in the Congress and excursion, were held back by financial considerations. Lipman was able to arrange for the expenses of the entire excursion through the United States and Canada, which lasted from June 22 to July 22, 1927, for all foreign participants, to be paid out of private funds. This fact contributed very largely to the success of the First Congress.

The results and benefits of the First Congress were briefly summarized, as follows, by Alexius A. J. de Sigmond:[1] (1) a large number of American scientists became interested in the Society, whereas previously few had participated in the conferences of soil science; (2) the delegation of 20 soil experts from Russia had the opportunity to develop in detail the results of the Modern Russian school of soil science; (3) membership in the Society reached the unprecedented number of 934. Among the members were scientists from all parts of the world where soil science was receiving consideration.

Lipman's bearing was invariably characterized by great simplicity; he was averse to all ostentation. I experienced this immediately on his first appearance as President of the International Society of Soil Science, on April 8, 1926, when he presided over the first meeting of the General Committee of our Society at Groningen.[2] I had expected that he would take this opportunity to make a more or less formal opening speech, in which he would expound the aims of the young Society. Nothing of the kind. "The meeting is opened; please, Dr. Hissink, what is the first point on the agenda?" That was all. And later, at the numerous meetings which he conducted in Washington in 1927, the same simplicity was noticeable.

Lipman always evinced a keen interest in our Society. In the last few years he zealously urged the holding of a Conference, if possible even an

[1] *Soil Sci.*, 40: 85.
[2] *Mitt. Internatl. Bodenk. Gesell.*, I/II (1925/26): 200.

International Congress, in one of the countries of South America. Our last correspondence was on this point.

Lipman was well known not only for his work on behalf of the International Society of Soil Science, but also as Dean and Director of the New Jersey Agricultural Experiment Station. In the midst of this busy career he found time not only to pursue his research activities, but also to serve as editor-in-chief of *Soil Science*, a journal which he founded in 1916.

And now Lipman has left us. Since the days at Prague many have preceded him. I think of Ramann, Frosterus, Glinka, Gedroiz, Wiegner, and others. Perhaps I may best conclude this tribute to Lipman by repeating the words of A. G. McCall in *Science*:[3] "Those of us who were fortunate enough to obtain glimpses of his wholesome and well-balanced philosophy of life at various times and under different circumstances are fortunate. A young scientist who has not had the friendship of a man of Dr. Lipman's type is spared the grief that comes with his loss, but his life is lacking one of its greatest joys and the satisfaction that comes out of such associations."

[3] *Science*, 89: 378-379, 1939.

MECHANISM OF SYMBIOTIC NITROGEN FIXATION BY LEGUMINOUS PLANTS*

ARTTURI I. VIRTANEN

Biochemical Institute, Helsinki, Finland

SYMBIOTIC nitrogen fixation and its chemical mechanism have been investigated in our laboratory for many years.† The most important problem in the elucidation of symbiosis is to find out, on the one hand, *the carbon compounds which the intranodular bacteria receive from the host plant in connection with the fixation of nitrogen in the root nodules*, and, on the other hand, *the nitrogen compounds which the host plant in its turn receives from the root nodules*. To elucidate the second question we carried out comparative experiments with legumes which, in some experiments, were uninoculated and received different nitrogen compounds for their nitrogen nutrition and which, in others, were growing without nitrogen fertilization but were inoculated with effective root nodule bacteria. It was proved that red clover grew noticeably better when inoculated but without nitrogen fertilization than when uninoculated but supplied with nitrates. The growth of

TABLE 1

GROWTH OF RED CLOVER IN QUARTZ SAND

pH 6.5; 10 plants in each pot; age of plants 106 days

N-nutrition	Inoculation	Dry weight of plants
		gm.
KNO_3	Not inoculated	23.78
KNO_3	Not inoculated	24.07
$(NH_4)_2SO_4$	Not inoculated	22.32
$(NH_4)_2SO_4$	Not inoculated	18.00
N-free medium	Inoculated	31.38
N-free medium	Inoculated	30.27

* Literature up to the end of 1937 is included in my book: *Cattle Fodder and Human Nutrition with Special Reference to Biological Nitrogen Fixation*, Cambridge, 1938. Of the later publications from our laboratory the following may be mentioned: Virtanen and Laine, *Nature* 141: 748 (1938); 142: 165 (1938); *Biochem. Jour.* 33: 412 (1939).

† My chief collaborators in these investigations have been Synnöve v. Hausen (now Mrs. Saubert) and T. Laine.

peas was, in both cases, about equally good. Table 1 illustrates the results of one of our experimental series with red clover.

The results shown in Table 1 could be interpreted by assuming either that red clover received from its root nodules some nitrogen nutrition more advantageous than nitrate or that a specific growth factor was formed in the root nodules. To examine the first alternative we investigated the ability of the legumes to utilize organic nitrogen compounds, especially different amino acids, for their nitrogen nutrition. For this purpose it was necessary to use a sterile culture system in the plant experiments, since in ordinary pot cultures organic nitrogen compounds are decomposed by microorganisms present in the medium, and consequently, ammonia and nitrate nitrogen will constitute the actual nitrogen nutrition. By means of the sterile culture system developed by us, we were able to ascertain that, of the amino acids investigated, *aspartic acid supplied the best nitrogen nutrition for legumes.* The effect of glutamic acid also was good, but other amino acids were utilized poorly or not at all. Red clover grew better on aspartic acid than on nitrate, and peas did almost equally well on both. It was very remarkable that the nonlegumes, barley and wheat, were virtually unable to utilize aspartic acid for their nitrogen nutrition. Table 2 clearly shows this point.

TABLE 2

GROWTH AND NITROGEN CONTENT OF RED CLOVER, PEAS, BARLEY, AND WHEAT PLANTS IN QUARTZ SAND (STERILE SYSTEM)

pH 6.5; one plant in each suction flask

N-nutrition	Dry weight	N in plant
Red clover	*gm.*	*mgm.*
KNO_3	2.329	50.0
Aspartic acid	4.428	90.9
Without N-nutrition	0.028	0.14
Peas		
KNO_3	1.402	40.1
Aspartic acid	1.474	40.0
Without N-nutrition	0.325	6.2
*Barley**		
KNO_3	0.433	13.3
Aspartic acid	0.049	1.9
Without N-nutrition	0.063	0.7
Wheat		
KNO_3	2.143	35.3
Aspartic acid	0.113	3.7
Without N-nutrition	0.117	0.8

* Culture of barley harvested at much earlier stage than other cultures.

The ability of the legumes—in contrast to the nonlegumes—to utilize aspartic acid in preference to other forms of nitrogen for their nitrogen nutrition and the observation that certain legumes, such as red clover, grew better when inoculated than when supplied with nitrates and ammonium salts led to the assumption that the legumes probably receive their nitrogen nutrition from the root nodules in the form of amino acids, possibly in the form of aspartic acid. This assumption was in good agreement with observations made somewhat later on *the excretion of nitrogen compounds from the root nodules.*

The excretion phenomenon was conclusively proved in 1930, when we ascertained that in inoculated cultures of vetch and peas growing in a sterile system, considerable amounts of nitrogen compounds were transferred to the medium. In view of the fact that no foreign bacteria were present and that free-living legume bacteria are not able to fix nitrogen, the nitrogen compounds present in the medium must originate from the root system of legumes and, in my opinion, from the root nodules. That the excretion really takes place *from the root nodules* and *not from the roots* was later proved in many different ways. For instance, it was noted that no organic nitrogen compounds are excreted into the medium of uninoculated peas growing on nitrates in a sterile culture system. The most convincing proof was obtained by the following experiment:

A sand-filled glass tube was sunk into quartz sand in a sterile culture of peas. The sand in the tube alone was inoculated with legume bacteria. Nodulation therefore occurred only in the roots which grew into the small

TABLE 3

EXCRETION OF NITROGEN FROM ROOT NODULES OF PEAS GROWN IN QUARTZ SAND (STERILE SYSTEM)

3-l. Woulff's bottles; 3.4 kgm. dry quartz sand; 3-l. N-free nutrient solution; pH 6.5. Three Torsdag peas in each bottle, inoculated with strain H X, all planted September 20, 1934, but harvested at different times: I, harvested October 24, before flowering; II, harvested November 1, at full bloom; III, harvested November 15, when ripe; IV, uninoculated control, harvested November 15

	Dry weight	Total N*	Excreted N in sand†	Total fixed N	Extent of excretion
	gm.	*mgm.*	*mgm.*	*mgm.*	*per cent*
I.....	2.149	50.0 [26.1]	66.8	92.9	71.9
II.....	4.370	95.1 [71.2]	89.0	160.2	55.2
III.....	6.163	143.4 [119.5]	99.7	219.2	46.0
IV.....	1.989	23.9	....	....	....

* The figures in brackets are those remaining after subtraction of controls.
† After subtraction of the initial N in sand.

glass tube. When the culture was harvested, an increase of nitrogen compounds could be noted only in the sand in the glass tube. This nitrogen was in the amino form (cf. below). In the quartz sand outside the tube where the major part of the roots grew without any nodules, no amino nitrogen could be detected.

Theoretically, it is of great value that the excretion of amino nitrogen has been proved to take place from the root nodules, and consequently, to be related to nitrogen fixation. The excretion of nitrogen compounds from the root nodules has been enormous in many of our experiments: in the most outstanding cases over 80 per cent of the total fixed nitrogen has

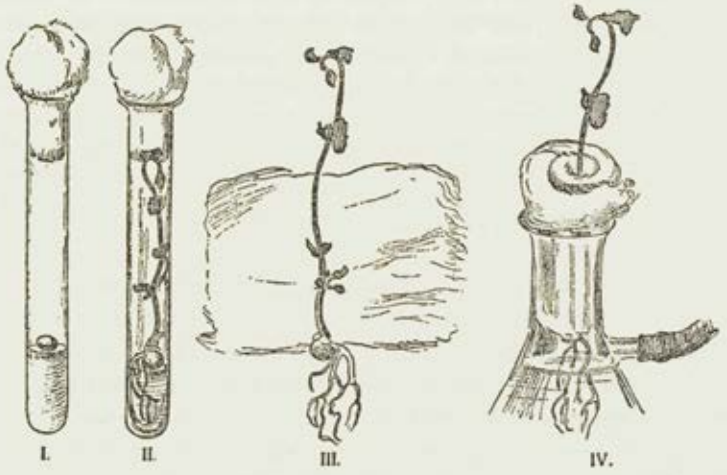


FIG. 1. Preparation of sterile culture. I. Sterilized seed on the surface of sterile agar. II. Sterile plant grown on agar in the tube. III. Plant removed from the tube, on sterile cotton. IV. Plant wrapped in cotton and transferred into a culture flask.

been excreted to the medium and less than 20 per cent utilized by the host plant. The excretion is most vigorous from young root nodules, as illustrated in Table 3.

In view of the large quantity of nitrogen excreted as well as the vigor of excretion, especially by young root nodules, it did not seem likely that the excreted nitrogen compounds were products of protein autolysis occurring in the root nodules. More natural was the explanation that the excretion products consisted of substances which were formed during nitrogen fixation but were not yet utilized for protein synthesis. Determination of the nature of these nitrogen compounds seemed likely to provide an answer to this question and, at the same time, in case these compounds proved to be primary products of nitrogen fixation, to offer a possibility for experimental investigation of the chemical mechanism of nitrogen fixation.

In order to isolate the excreted nitrogen compounds, it was, naturally, necessary to grow inoculated legumes under sterile conditions, since otherwise excretion products would be decomposed by different microorganisms. Figure 1 illustrates our technic of sterile culture.

This experimental system has been very satisfactory in that infection by foreign microorganisms has been rare. The transfer of the sterile plant from the tube to the actual culture flask was carried out in a special room, the air in which was first sterilized by means of a quartz lamp. As the person who transferred the plants wore a sterilized overall, a cap, and rubber gloves, the sterile transfer of the tender plant succeeded almost without exception. The inoculation of the seed with pure cultures of legume bacteria was carried out with a platinum wire. For medium in our experiments we have generally used nitrogen-free quartz sand. From finely divided cellulose, which was later tried, extraction of the excreted nitrogen compounds is easier than from sand. In general, however, excretion is more pronounced in sand, for sand absorbs the excreted nitrogen compounds more readily than does cellulose. As we have shown, it is essential that the medium be able to absorb the excreted products. The excretion in water cultures is so weak, therefore, that it can hardly be noted.

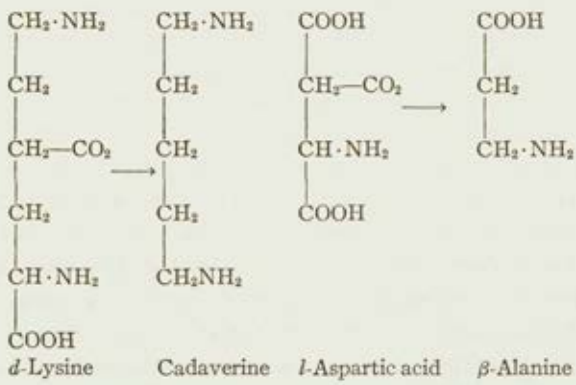
After harvest of the culture, the roots were carefully removed from the sand, which was then thoroughly extracted at room temperature with water acidified with sulfuric or hydrochloric acid to about pH 4. In this manner, only 70-80 per cent of the total excreted nitrogen generally was recovered in the solution. Lately, however, we have recovered almost 100 per cent. From cellulose the nitrogen compounds can be quantitatively extracted with two washings. The water extract obtained is then evaporated *in vacuo* to a small volume, after which the concentrate is ready for investigation.

The determination of amino nitrogen, according to the Van Slyke method using 30 minutes' reaction time, showed that the extract from different pea cultures contained 90-98 per cent of the total nitrogen in this form. When the time of reaction was 5 minutes, the amount of amino nitrogen was smaller, a fact which implies that a part of the amino nitrogen is in a position other than the α with regard to carboxyl. Formaldehyde titration led to a similar result. Qualitative experiments showed that the solution contained neither α-amino-monocarboxylic acids nor aromatic amino acids. The number of amino acids present in the extract thus was very limited.

If the culture of inoculated peas is harvested in the beginning of flowering, about 50 per cent of the nitrogen in the extract obtained from the sand precipitates from the alcoholic solution as calcium or barium salts, according to Foreman. The Foreman fraction consists of *l-aspartic acid*, which can be isolated as copper salt and determined quantitatively by means of the

enzyme aspartase. Glutamic acid, which also precipitates, according to Foreman, was not found in the extract.

The rest of the amino nitrogen was precipitated with phosphotungstic acid. It seemed, at first, that this nitrogen fraction must have consisted of lysine, according to the ordinary method for determination of diamino acids. Its isolation, however, failed. Later we succeeded in establishing that the fraction did not contain lysine, since a certain strain of coli bacteria, which decarboxylates lysine quantitatively to cadaverine, did not bring about the formation of cadaverine. Soon afterward, we discovered that legume bacteria split off one carboxyl group from *l*-aspartic acid with the formation of β-alanine.



d-Lysine Cadaverine *l*-Aspartic acid β-Alanine

We could also show the presence of β-alanine in the phosphotungstic acid fraction, which probably consists exclusively of this particular amino acid. With the ninhydrin reaction, the nitrogen fraction and β-alanine give about the same amounts of acetaldehyde. The nitrogen fraction in question also dissolves quantitatively in absolute methyl alcohol. A reliable method for the quantitative determination of β-alanine has not yet been found.

These investigations supported the view that the root nodules excrete chiefly *l*-aspartic acid and that the other amino acid, β-alanine, found among the excretion products is only a secondary decomposition product of *l*-aspartic acid. The excreted amino nitrogen thus consists primarily of aspartic acid nitrogen. In view of the fact that in the most favorable cases the amino nitrogen, consisting of aspartic acid nitrogen excreted from young root nodules, amounts to about 80 per cent of the total fixed nitrogen, it is evident that *the excreted nitrogen compounds cannot be products of protein breakdown.* The protein of the root nodules contains, besides many other amino acids, only about 10 per cent aspartic acid, and the nitrogen in the root nodules amounts only to 1 or 2 per cent or even less of the total fixed nitrogen. *Aspartic acid thus represents the primary amino acid which is*

formed in the biological fixation of nitrogen, and has not yet been utilized in the protein synthesis.

This result was noteworthy in connection with the earlier observation that aspartic acid is an excellent source of nitrogen for legumes. We have every reason to assume, therefore, that *aspartic acid is the particular nitrogen compound which the host plant takes up from the root nodules for its nitrogen nutrition.* The fact that many legumes in symbiosis with efficient nodule bacteria attain excellent growth is thereby afforded a plausible explanation. The legumes thus receive their normal nitrogen nutrition in quite a different form from that of the nonlegumes, which chiefly use nitrates and ammonium salts. Whether the legumes continue to receive some specific growth factors from their root nodules is hypothetical. The occurrence of aspartic acid in the process of nitrogen fixation seems to explain sufficiently the favorable influence of symbiosis on the growth of the host plant. Possibly β-alanine may also strengthen the influence of aspartic acid, but this fact has not yet been experimentally proved.

After aspartic acid was established to be the primary amino acid in biological nitrogen fixation, the following step in the elucidation of the mechanism of nitrogen fixation concerned the formation of this acid. Two reactions were known heretofore to lead to the biological formation of aspartic acid; first, that from ammonia and fumaric acid, and second, that from ammonia and oxaloacetic acid through the imino acid stage. The former alternative was eliminated after we found that neither legume bacteria nor root nodules contained the enzyme aspartase. The latter alternative also was unlikely because ammonia could not be detected among the excretion products.

Our attention was then directed to a third possibility, viz., that *hydroxylamine forms with oxaloacetic acid an oxime, which is then reduced to aspartic acid.* In 1935 we noted the occurrence of NO_2-N in the extract of the medium of inoculated peas. The major part of NO_2-N appeared during the extraction and the concentration of the extract. Thus the medium must have contained some nitrogen compounds, from which the nitrite nitrogen is easily formed. At the same time Endres found small amounts of carboxime nitrogen in Azotobacter cultures. We were also able to find some oxime nitrogen, usually 1 or 2 per cent of the total nitrogen, among the excretion products of root nodules. The hypothesis which Blom had earlier presented of the formation of hydroxylamine as an intermediate product in nitrogen fixation, therefore, seemed possible. I wish, however, especially to point out that an occurrence of small amounts of some atom grouping containing nitrogen among the excreted products does not prove that the said atom grouping belongs to an intermediate product of nitrogen fixation. As ammonia

is easily formed through secondary decomposition products, oxime nitrogen can be thought to occur in a similar manner. Only when it is possible to isolate as reaction products well-characterized nitrogen compounds which are so related to one another that it is possible by means of them to follow step by step the course of reaction, can the compounds be regarded as intermediate products of nitrogen fixation. The discovery of the occurrence of oxime nitrogen among the excretion products gave direction to the elucidation of the mechanism of the formation of aspartic acid, but in no way explained the course of the reaction as long as it was not known which carboxime compound was in question.

In order to elucidate the mechanism of the formation of aspartic acid it was necessary to isolate and characterize the excreted oxime compound. Its isolation was very difficult, because of its small amount and especially of its ready decomposition during the process of isolation. Even in extraction and evaporation *in vacuo*, nitrite nitrogen is formed from the oxime. It is often found in small amounts in the original culture and is then formed also from oxime compound, as we were able to prove experimentally when the oxime was characterized.

The isolation of the oxime compound was accomplished in the following way: The concentrate of the excreted nitrogen compounds was extracted with ether, in which the oxime compound dissolves. In this manner the oxime was separated from *l*-aspartic acid and β-alanine. The ether extract was dissolved in water and precipitated according to the method of Foreman, whereby the oxime nitrogen was entirely precipitated. This indicates that the compound was an oxime of some dicarboxylic acid. Further purification of the oxime was made difficult by the presence of fumaric acid, which also appears in small amounts among the excretion products. Eventually, however, we succeeded in isolating the oxime as a copper salt, the analysis of which proved that the compound consisted of *oximinosuccinic acid*. The oxime compound was later characterized also by reduction with hydrogen in the presence of finely divided platinum, whereby aspartic acid was formed. This was then easily separated from succinic acid formed on reduction from fumaric acid.

The following formulas present all the excretion products isolated by us:

THE EXCRETION PRODUCTS OF THE ROOT NODULES

NO_2 — Appears chiefly during extraction and concentration.

$HO_2C \cdot C(NOH) \cdot CH_2 \cdot CO_2H \longrightarrow HO_2C \cdot CH(NH_2) \cdot CH_2 \cdot CO_2H \longrightarrow CH_2(NH_2) \cdot CH_2 \cdot CO_2H$

Oximinosuccinic acid	Aspartic acid	β-Alanine
Generally 1–2 per cent of the excreted N.	Varying amounts depending on decarboxylation. In young cultures up to 75 per cent of the excreted N, in older ones only 20–30 per cent.	Amount depends on the decarboxylation of aspartic acid. Therefore more abundantly found in old than in young cultures.

l-Aspartic acid-N and β-alanine-N form together over 90 per cent of the total excreted N.

$HO_2C \cdot CH=CH \cdot CO_2H$

Fumaric acid

Small amounts among excretion products. Probably formed from oxaloacetic acid in the root nodules.

The occurrence of the oximinosuccinic acid as well as the great amount of aspartic acid among the excretion products can, in my opinion, be interpreted only by assuming that aspartic acid is formed over oximinosuccinic acid. Since our experiments have shown that hydroxylamine and oxaloacetic acid react instantaneously with each other even in the most dilute solutions without catalysts, there is reason to assume that hydroxylamine is formed in the process of nitrogen fixation and that oxaloacetic acid forms the oxime in question. The mechanism of biological nitrogen fixation thus would be as follows:

N_2 (atm. nitrogen) → NH (di-imide)? = NH

$C_6H_{12}O_6 \longrightarrow$ Carbohydrates in plants → Oxaloacetic acid (COOH–CO–CH_2–COOH) + NH_2OH (Hydroxylamine) → Oximinosuccinic acid (COOH–CNOH–CH_2–COOH) → *l*-Aspartic acid (COOH–$CHNH_2$–CH_2–COOH)

The fact that hydroxylamine, as such, could not be found as an intermediate product in nitrogen fixation is easy to understand in view of the rapid reaction of hydroxylamine with oxaloacetic acid. In this connection it must be mentioned that the reaction between hydroxylamine and other keto acids, e.g., ketoglutaric and pyruvic acids, is appreciably slower than that between hydroxylamine and oxaloacetic acid.

The reduction of oxime to aspartic acid is brought about readily by legume bacteria and leguminous plants. When oximinosuccinic acid and glucose are added to the heavy suspension of legume bacteria, *l*-aspartic acid is formed, according to our experiments. In leguminous plants oximino-succinic acid is also used in great dilution as N-source. The hydrogen required for the reduction is evidently derived from sugar always found in plants.

Of the later fate of aspartic acid in the host plant and of the formation of other amino acids the following facts are known: Braunstein and Kritzmann, in 1937, reached the important conclusion that in animal tissues only the amino group of aspartic and glutamic acids can be utilized for the formation of other amino acids from keto acids. Accordingly, aspartic and glutamic acids have a special position in the synthesis of amino acid in the animal organism. Somewhat later we were able to establish that if *l*-aspartic acid and pyruvic acid are transferred to a water suspension of crushed pea plant a transamination takes place rapidly, and as a result, α-alanine and oxaloacetic acid are formed. On the other hand, no phenyl alanine is formed under the same conditions from α-alanine and phenyl pyruvic acid. Aspartic acid, therefore, acts as an intermediary for the formation of other amino acids from the respective keto acids. We have thus succeeded in following step by step the synthesis of amino acid from atmospheric nitrogen. The formation of aspartic acid as a primary amino acid is also understandable, as other amino acids can be formed through the intermediary of aspartic acid.

It is noteworthy, although somewhat surprising, that glutamic acid is not formed during nitrogen fixation by the root nodules, in spite of the fact that glutamic acid together with aspartic acid has a special position in the synthesis of amino acid. In Von Euler's laboratory in Stockholm important investigations were carried out last year concerning the specific glutamic acid dehydrogenase which generally occurs also in microorganisms and plants. As the intermediate product of biological nitrogen fixation is not ammonia but hydroxylamine, which reacts more vigorously with oxaloacetic than with ketoglutaric acid, the formation of aspartic acid as the only primary amino acid in the process of nitrogen fixation is explicable.

The expenditure of organic substance in the fixation of nitrogen, calculated per nitrogen unit, is appreciably less in the symbiotic process than is that by the free-living Azotobacter, by which nitrogen fixation is connected with the growth and multiplication of cells, and consequently, fixed nitrogen is

chiefly found in the protein of the bacteria cells. On the other hand, the intranodular bacteria excrete the major part of the fixed nitrogen, a part of which is taken up by the host plant, the other part being diffused from the root nodules into the medium. Hence it follows that while Azotobacter fixes about 20 mgm. nitrogen per 200 mgm. of bacteria, calculated as dry matter, the intranodular bacteria are able to fix quite different quantities of nitrogen. In one of our experiments, for example, in which 190 mgm. of nitrogen was fixed per pea plant, the weight of the root nodules of that plant was 60 mgm., calculated as dry matter. Since only a part of the root nodules consists of bacterial mass, it can be concluded that about 100 times more nitrogen was fixed by intranodular bacteria per weight unit than by Azotobacter.

Since in symbiotic nitrogen fixation the bacterial cells do not store appreciable quantities of fixed nitrogen in the form of proteins, the uptake of carbon compounds is naturally very small compared with that by the free-living nitrogen-fixers. Nitrogen fixation can be assumed to occur on the surface of the intranodular bacteria whereby the hydroxylamine formed reacts with the oxaloacetic acid present in the plant sap. The sugars, and possibly other compounds, in the host plant apparently act as hydrogen donators in the reduction processes taking place in the fixation of nitrogen. As nitrogen fixation is an exothermic reaction and the hydrogen originates from the host plant, the slight expenditure of carbon compounds in the fixation of nitrogen is easily understood.

The important role of the oxaloacetic acid in the fixation of nitrogen has been confirmed synthetically. The earlier attempts to bring about a distinct nitrogen fixation in sugar solutions with free-living legume bacteria or with excised root nodules had failed. About two years ago we succeeded, however, in showing *distinct and, in many instances, vigorous nitrogen fixation with excised root nodules in a dilute neutral solution of oxaloacetic acid* within a few hours. Two different methods were used in our experiments for the determination of nitrogen fixation, viz., the determination of fixed nitrogen by the Kjeldahl method in the suspension of excised nodules, on the one hand, and the determination of the decrease of nitrogen in the gas volume in a closed reaction system, on the other. We have employed the technic illustrated in Figure 2, by means of which several parallel determinations can be made.

The accuracy of this apparatus in the measurement of gas is such that a decrease of as little as 0.2 cc. of nitrogen gas can be detected. In the control experiment with root nodules in water without any substrate the volume of nitrogen does not decrease. In the presence of oxaloacetic acid the volume of nitrogen decreases to such an extent that the errors of measurement are excluded. At the end of the experiment fixed nitrogen can be determined

also in the reaction suspension by the Kjeldahl method, thus establishing the increase of nitrogen. In the series of 25 experiments hitherto carried out with root nodules of peas, all experiments with oxaloacetic acid produced distinct nitrogen fixation. With succinic acid nitrogen fixation did not occur.

In the experiments with excised root nodules in the presence of oxaloacetic acid, the major part of the fixed nitrogen is transferred into the surrounding

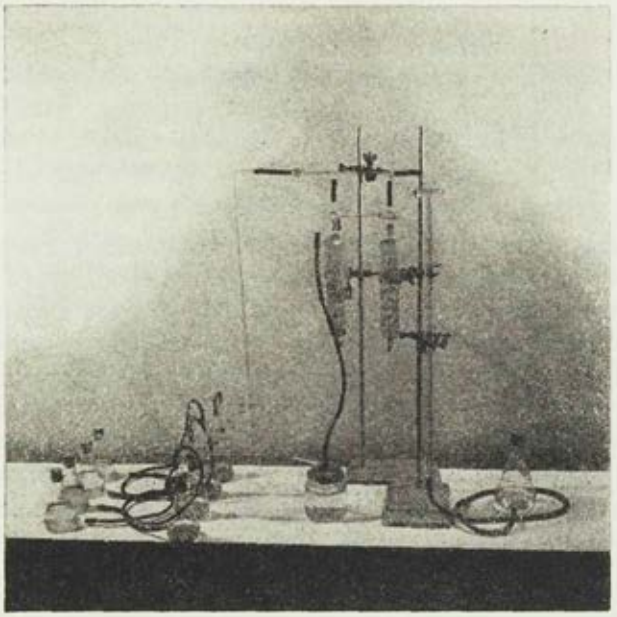


FIG. 2. Orsat's gas burette and absorption flask are connected with Erlenmeyer flasks, which, in turn, are connected with other Erlenmeyer flasks. The latter ones contain so much substrate solution that the air in the experimental flasks can be driven out by means of the solution in the beginning of the experiment. After that an exactly measured volume of air (100.0 cc.) is pressed from the gas burette to the experimental flasks. After the experiment the air volume is again passed from the experimental flasks to the gas burette, and pressed further into the absorption flask, in which the oxygen and the carbon dioxide are absorbed in an alkaline sodium hydrosulfite solution ($Na_2S_2O_4$). After this the total volume of the rest of nitrogen is read.

solution in the form of amino nitrogen. Oxime nitrogen can also be shown in the solution. This fact is analogous to the excretion by the nodules in the roots of living plants. The results of one of our experiments are shown in Table 4.

In the control experiment without oxaloacetic acid, when no nitrogen fixation takes place, amino nitrogen increases somewhat in the solution because cells are broken on the cut surface. The difference between the amounts of amino nitrogen in the control experiment and in the experiment

TABLE 4

NITROGEN FIXATION BY EXCISED ROOT NODULES

In each experiment 9.5 gm. of root nodules of pea in 50 cc. of solution; pH 7. I. Without oxaloacetic acid; II. With 200 mgm. of oxaloacetic acid

	Amino-N in the solution		Oxime-N in the solution after 5 hours
	At the start	After 5 hours	
	mgm.	*mgm.*	
I	0.0	1.3	nil
II	0.3	9.0	distinct reaction

with oxaloacetic acid is, however, very great. No oxime nitrogen is formed in the control experiments, a fact that deserves full attention.

In view of all the foregoing facts, oxaloacetic acid has a central position in the biological fixation of nitrogen. We have therefore investigated its appearance in legumes. In numerous experiments with growing peas and red clover, we have been able to show the occurrence of oxaloacetic acid in appreciable quantities. Accordingly, the intranodular bacteria obtain this central keto acid from the plant sap. When peas have developed thick pods and nitrogen ceases to increase, oxaloacetic acid is no longer found.

The results obtained in connection with the biological fixation of nitrogen throw light also on the synthesis of amino acids when the plants are using nitrate and ammonium nitrogen for their nitrogen nutrition. When plants receive nitrates as nitrogen nutrition, amino-acid synthesis is evidently similar in principle to that in the assimilation of atmospheric nitrogen. Under favorable conditions, nitrite nitrogen is formed to some extent in plants receiving nitrates. That nitrite nitrogen is not accumulated in greater amounts is easily understood on the basis of the observations made by Lemoigne and Michlin, according to which ascorbic acid and also glutathione rapidly reduce nitrites to hydroxylamine. The occurrence of carboxime nitrogen in small amounts in Azotobacter cultures which receive nitrates for nitrogen nutrition also supports the formation of hydroxylamine.

When ammonium salts form the nitrogen nutrition of plants, the amino acid synthesis occurs apparently through the intermediary of ketoglutaric acid and oxaloacetic acid through the corresponding imino acids, with glutamic and aspartic acid as end products. These reactions are catalyzed by specific glutamic and aspartic acid dehydrogenases. Another reaction proceeds from ammonia to amino acid through fumaric acid, with aspartic

acid as end product. The enzyme aspartase which catalyzes this reaction is often found, especially in microorganisms.

The difference in the amino acid synthesis in the biological fixation of nitrogen when nitrates form the nitrogen nutrition of plants, on the one hand, and when ammonium salts are used as the source of nitrogen, on the other, is a noteworthy fact in regard to the nitrogen nutrition of plants. The advantageous position of nitrate nitrogen for many plants can thus be understood.

ADDENDUM

After I had written the foregoing paper, volume 8 of "Ergebnisse der Enzymforschung" appeared containing an article by P. W. Wilson on "Mechanism of Symbiotic Nitrogen Fixation." The following comments are evoked by this article:

Wilson seems to have little confidence in the methods of organic chemistry in the elucidation of biological nitrogen fixation. In my opinion, the chemical mechanism of nitrogen fixation cannot be discovered by determining the dependence of nitrogen fixation upon the pressure of nitrogen, the pressure of oxygen, or the presence of hydrogen, whereas Wilson is of the opinion that the classical methods for investigation of biochemical mechanism, e.g., the isolation of intermediates, are not applicable to the elucidation of the chemistry of nitrogen fixation. He writes,

> Because of the numerous and diverse reactions which take place in the development of a plant, isolation of any specific compound postulated in a given hypothesis may not be particularly difficult. Such isolation, however, lends little convincing support to that hypothesis unless it is demonstrated that the compound is unique for the fixation reaction, i.e., that the manner of its occurrence in the plant depends upon the activity of the fixation system. Since this demonstration is usually lacking, present information based on the "organic chemistry" approach provides little more than suggestive leads whose validity remains to be established.

In writing the foregoing statement, Wilson has apparently disregarded the fact that it has been proved—in my opinion, convincingly—in our laboratory that the excretion products are compounds diffused from the root nodules to the medium, as described in the foregoing paper. *These compounds do not appear in the medium when the legumes are grown with nitrate fertilization* or *without root nodules.* The nitrogen compounds we have isolated thus are "unique for the fixation reaction," i.e., "their occurrence in the plant depends upon the activity of the fixation system."

Wilson considers it quite possible that any aspartic acid excreted is a product of protein metabolism of the plant and not, as we regard it, an amino acid which has not yet been used in the protein synthesis. Wilson supports his conception by the observations of Greenhill and Chibnall that if large quantities of ammonium sulfate are supplied to rye grass, the excess

nitrogen is converted into glutamine and excreted from the leaves. On the basis of our present knowledge this observation in no way contradicts our idea of the formation of aspartic acid as a primary amino acid in the fixation of nitrogen; but, on the contrary, is in full agreement with it. *Aspartic and glutamic acids are the fundamental amino acids from which the amino group is transformed to other keto acids.* Glutamic acid is formed from ammonia and ketoglutaric acid through the effect of glutamic acid dehydrogenase. If fertilization with ammonium salts is very heavy, the synthesis of glutamic acid increases to such an extent that other amino acids cannot be formed from glutamic acid at the same rate. The excess glutamic acid may then be excreted from the leaves to some extent in the form of glutamine. Glutamic acid is, in this case, the primary amino acid.

Wilson expresses the opinion that if other amino acids, instead of aspartic acid, were excreted from the root nodules he would consider their position as intermediate products more convincing than the present case. Wilson's idea is entirely at variance with my conception, since only aspartic and glutamic acids may come into question as primary amino acids. This fact was not yet known in 1935-36 when we found aspartic acid as an excretion product of root nodules and the present idea was advanced of the mechanism of nitrogen fixation; but on the basis of our present knowledge of amino acid synthesis, the formation of aspartic acid as the primary amino acid is only to be expected. That glutamic acid is not formed seems to be ascribable to the fact that the reaction velocity of hydroxylamine with oxaloacetic acid is many times greater than with ketoglutaric acid. Should nitrogen fixation occur through the ammonia stage, the formation of glutamic acid together with aspartic acid would be very likely.

In his article, Wilson also presents some of his experiments, the results of which differ from those obtained in our laboratory. He reports that he has not been able to find the slightest amount of oxaloacetic acid in the legumes. As was shown in my communication to *Nature* (in press) Wilson's negative result is apparently due to the methods employed. If alkali is added to the plant material to stabilize the oxaloacetic acid before the plants are crushed and the sap pressed off, the oxaloacetic acid is preserved in the sap and can be determined by means of the CO_2 formed through the effect of aniline. By this procedure varying amounts of oxaloacetic acid are found in well-growing legumes.

Wilson reports also that he has not been able to bring about nitrogen fixation with excised root nodules in oxaloacetic acid solution. This result is in contradiction to numerous experiments carried out by us. As we have determined the amount of fixed nitrogen by the Kjeldahl method and, in addition, the decrease of nitrogen gas in the gas mixture, I do not see

that there can be any doubt in regard to our results. Wilson has determined the percentage of oxygen in the gas mixture and assumes it to increase when nitrogen is fixed. In view of the fact that the root nodules respire and consume oxygen during the experiment, I consider the determination of the decrease of nitrogen in the entire volume of gas as the only reliable method of determining nitrogen fixation by means of gas analysis.

A SUGGESTED EXPLANATION OF THE "INEFFICIENCY" OF CERTAIN STRAINS OF RHIZOBIA

H. G. THORNTON

Rothamsted Experimental Station, Harpenden, England

WITHIN those cross-inoculation groups that have been extensively studied, strains of nodule bacteria are now known that show every grade of efficiency down to that where there is no visible benefit on the host plant and where fixation of nitrogen is scarcely appreciable. It seems doubtful, however, whether any of the strains so far known fail *entirely* to increase the nitrogen of the plants upon which they have formed nodules.

It has been a natural hypothesis that the inefficient strains were physiologically deficient in nitrogen-fixing ability, but this hypothesis has remained unsupported, because no strain of nodule bacterium can certainly be shown to fix nitrogen outside its host plant.

The Bacteriology Department at Rothamsted has therefore been attacking the problem of inefficient strains from a rather different angle, namely, by comparing the structure of nodules produced by efficient and inefficient strains in order first of all to ascertain if any differences in structure were correlated with efficiency or its opposite. The work has been carried out by Dr. Hugh Nicol, Mr. H. K. Chen, and the author in collaboration. The investigation was commenced with clover nodules, this choice being dictated first by the agricultural importance of clover and secondly by the fact that highly inefficient strains of clover nodule bacteria have been isolated. Our material was obtained by inoculating clover, usually red clover, with the highly efficient strains *205*, *A* and *W*, and with the inefficient strains *202* and *Coryn*. Strains *205* and *202* were kindly supplied to us by Wisconsin Agricultural Experimental Station, and strain *A* by Professor Barthel of Stockholm. The other strains were isolated at Rothamsted. Most of the nodules studied came from plants grown in agar but the results were checked on material from sand cultures.

ANATOMY OF EFFICIENT AND INEFFICIENT CLOVER NODULES

Inefficient clover nodules differ from efficient nodules in their much smaller size and this can be seen in sections to be due to the failure of the former to establish a persistent apical meristem cap. This results in a big difference

in the volume of the central tissue composed largely of swollen cells containing bacteria, here described as the "bacterial tissue." We have found that nodules produced by the efficient strain *A*, having a mean length of 1.5 mm., contain a mean volume of about 0.15 cmm. of bacterial tissue, whereas nodules produced by the inefficient *Coryn* strain, with a mean length of 0.5 mm. have a mean bacterial tissue content of only 0.03 cmm. There is therefore about 5 times as much bacterial tissue in the efficient as in the inefficient nodules.

But there is also a second distinguishing characteristic of the bacterial tissue, namely its persistence in time. In all nodules by whatever strain they are produced, the bacterial tissue eventually disintegrates. In clover nodules this disintegration commences at the base and works its way towards the apex of the nodule until all the central tissue has broken down. The disintegration is accompanied by a fragmentation and finally by a disappearance of the contained bacteroids. The age of nodules at which this process takes place is strikingly different with efficient and with inefficient nodules. In efficient nodules produced by strain *A* and *205*, the disintegration commences on an average in nodules 35 days old and is complete after 46 days. In inefficient nodules produced by the *Coryn* and *202* strains, disintegration is complete after 7 to 8 days.

Since the bacterial tissue contains nearly all the bacteria in the nodule we may regard it as the seat of nitrogen fixation. In efficient clover nodules this tissue is thus not only some five times as large as in inefficient nodules, but it is active for about six times as long.

Some nitrogen determinations on clover grown in bottles containing sand have enabled us to form some estimate of the effects of these factors (table 1). In this experiment the efficient strain *A* produced 122 nodules per bottle (mean of 23 bottles); the inefficient strain *Coryn*, 771 nodules (mean of 12

TABLE 1

NODULATION AND NITROGEN FIXATION BY EFFICIENT AND INEFFICIENT STRAINS

	Strain *A*	Strain *Coryn*
Number of replicates	23	12
Nodules per bottle	122	771
Bacterial tissue per bottle (cmm.)*	17.39	15.27
N-fixed per bottle (mgm.)	6.6	1.5
N-fixed per 100 cmm. of bacterial tissue (mgm.)	37.9	9.8
Duration of healthy bacterial tissue (days)	46	8
N-fixed per 100 cmm. bacterial tissue per day (mgm.)	0.82	1.23

* There is a definite relationship between the overall nodule length and the volume of bacterial tissue in clover nodules. This relationship was used in calculating the bacterial tissue volume from frequency curves of nodule lengths.

bottles). The bacterial tissue calculated per bottle was 17.4 cmm. for the strain *A*, and 15.27 cmm. for the more numerous but much smaller *Coryn* nodules. The nitrogen fixed per bottle for strain *A* was 6.6 mgm. and for *Coryn* 1.5 mgm. This means that 100 cmm. of strain *A* bacterial tissue fixed 37.9 mgm. of nitrogen and the same mass of *Coryn* bacterial tissue fixed 9.8 mgm. But if the strain *A* bacterial tissue was active for 46 days and the *Coryn* bacterial tissue for only 8 days, the nitrogen fixed by 100 cmm. of bacterial tissue per day was 0.82 mgm. for strain *A* and 1.23 mgm. for *Coryn*. The calculations are, of course, so approximate that we cannot say whether these figures are significantly different, but they make it very doubtful whether the bacterial tissue in the inefficient nodule is any less active in fixing nitrogen than that in the efficient nodule. At any rate in the case of clover the small volume and short duration of the active tissue seems to be by far the most important factor in accounting for the small nitrogen fixation by inefficient strains.

We have also made a preliminary study of the anatomy of pea and soy bean nodules produced by some of the efficient and relatively inefficient strains, isolated and kindly supplied to us by the Wisconsin Agricultural Experiment Station. In peas there is also a difference in mean length and consequently in bacterial tissue content as between efficient and inefficient nodules. In soy beans, the mean diameters do not differ markedly but the ratio of infected to uninfected cells in the bacterial tissue is about twice as large in efficient as in inefficient nodules. Both in peas and soy beans there is again a striking difference in the period of survival of the bacterial tissue as between nodules produced by efficient and inefficient strains.

Thus in clover, peas, and soy beans inefficient nodules are characterised by a small number of infected cells which disintegrate after an abnormally short period. The disintegration of the bacterial tissue has been studied in the past at Rothamsted in the case of lucerne and clover nodules produced by efficient strains and it has been shown that it can be accelerated by changes in the physiology of the host plant This suggests the possibility that a similar unfavourable reaction may be induced in a host plant that is infected by an inefficient strain.

We have obtained some experimental evidence that this is the case. Peas and soy beans infected with efficient and inefficient strains, as well as uninoculated ones, were grown in sand, and the root juice from each set was extracted and sterilized by filtration. These filtered root juices were added to culture media known to be otherwise suitable for the growth of the nodule organism, and these media were inoculated with various strains of pea and soy bean nodule bacteria. The media containing root juices from plants bearing inefficient nodules produced a smaller growth of the bacteria than did the media containing juice of uninoculated roots or juice from roots of plants

TABLE 2

GROWTH OF THREE STRAINS OF PEA RHIZOBIA ON AGAR MEDIA CONTAINING ROOT JUICE FROM VARIOUSLY INOCULATED PEAS

Bacterial strains tested		Agar medium with root juice from following				
		Uninoculated peas	Peas bearing nodules by bacterial strains*			
			310	*317*	*313*	*B33*
317	Mean colony area, sq.mm.	153.0	114.2	154.3	103.4	111.4
	s.d.	±6.5	±4.7	±3.9	±4.9	±6.5
	n	28	39	39	37	28
313	Mean colony area, sq.mm.	105.3	103.2	156.6	92.5	94.2
	s.d.	±4.0	±4.4	±4.1	±3.6	±3.1
	n	29	39	39	39	39
B33	Mean colony area, sq.mm.	130.5	126.3	147.0	92.8	113.5
	s.d.	±5.1	±6.6	±4.5	±4.9	±3.4
	n	39	37	38	39	39

* Strains 310 and 317 are efficient, strains 313 and B33 inefficient.

bearing efficient nodules. The data from one of these experiments are presented in table 2.

It seems possible, therefore, that certain strains of nodule bacteria owe their inefficiency to the induced production by the host plant of a substance harmful to the bacteria and that the presence of this substance in the nodules inhibits the growth of the bacteria and causes their early disintegration.

THE PHYSIOLOGY OF NODULE FORMATION

KENNETH V. THIMANN

Harvard Biological Laboratories, Cambridge, Mass., U. S. A.

IN 1936 the author put forward the theory that the development of nodules was due to the formation of auxins by the invading bacteria. It has often been pointed out that cells in the root surrounding the zone of infection, but not themselves infected, are stimulated to division, i.e., that some diffusible substance must be concerned.

It is known from previous work[6] that the auxins, a group of substances distinguished by the similarity of their physiological action, though differing considerably in chemical structure, exert very characteristic effects on roots. Application of auxin promotes the formation of root initials but inhibits their subsequent elongation, causing instead a swelling of the root tissues, especially of the cortical layers; these layers show cells greatly shortened longitudinally, but with marked elongation in the radial direction. It was therefore suggested that the nodule is in essence a lateral root which is initiated but is not allowed to extend and hence swells into a more or less spherical structure. The initiation results from auxin which is produced by the bacteria and diffuses into the uninfected pericycle.

The author has already given evidence for the production of auxin within the nodule,[5] and Link[4] has adduced additional supporting facts. The present paper gives further material and summarizes the evidence which supports this view. The fact that substances other than auxin also play a part in nodule development is, of course, not excluded.

PRODUCTION OF AUXIN IN NODULES

Pisum sativum. Healthy, light-colored nodules, which were dissected from pea plants grown in soil and which were thoroughly washed, were sliced parallel to the root on which they were borne, and the halves placed upon agar (approximately 10 halves per block 75 sq. mm. in area). After a period of contact the blocks were cut into 12 small blocks, 10 cu. mm. in volume, and these were tested on etiolated *Avena* coleoptiles according to standard methods.[6] Both the apical and basal halves of the nodule were found to produce auxin in considerable amount, averaging 100-120 plant units per 10 nodules, when 3 hours of contact with the agar were allowed.

The maximum yield was obtained with nodules about 3 mm. in diameter. Older, brown nodules gave little if any auxin.

Phaseolus vulgaris. Similar experiments with nodules from bean plants have brought to light a characteristic difference between the behavior of nodules and that of non-pathological sources of auxin such as the coleoptile tips of *Avena*.

TABLE 1

Phaseolus vulgaris—Auxin in Plant Units per 10 Half-nodules

Transferred to fresh agar after each period				Continuous contact
1st hour	*2nd hour*	*3rd hour*	*Total in 3 hours*	*3 hours*
211	165	126	502	115
1st 10 mins.	*2nd 10 mins.*	*3rd 10 mins.*	*Total in 30 mins.*	*30 mins.*
118	100	68	286	102

Table 1 shows that the production of auxin by isolated nodules, when continually transferred to fresh agar, is maintained reasonably well with time, although it falls off somewhat, as might be expected. If, however, the nodules are left in contact with the same agar for longer periods, the concentration reached is no greater than that reached in the shorter time. This holds whether the contacts are 1 hour or 10 minutes in duration.

TABLE 2

Phaseolus vulgaris—Auxin in Plant Units per 10 Half-nodules

Time of contact with agar	Apical halves	Basal halves
20 seconds	53	38
5 minutes	53	19
30 minutes	65	53

The same relationship is shown in a different way in Table 2, which makes clear that the concentration reached is more or less independent of the time of contact. In other words, it seems probable that the agar merely comes into equilibrium with the auxin concentration in the nodule. The nodule thus behaves as a very large reservoir of auxin, much as a large agar block would. There is apparently no mechanism leading to polar conduction of auxin into the agar and its accumulation there, as would be the case with coleoptile tips.

A similar conclusion follows from the fact that the basal halves of the

nodules deliver auxin in amounts comparable with those given by the apical halves. The yields are somewhat smaller, but they show the same relationship with time (Table 2). If there were polar transport away from the tip of the nodule, auxin would not be expected to diffuse out of the lower half. This fact also supports the view that the auxin is produced by the bacterial tissue and not by the meristem, which of course would be in the apical half only.

Alnus glutinosa. Since the same mechanism should hold for nonleguminous plants as well as for leguminous plants, some experiments were carried out with nodules from alder.* Diffusions, carried out in the usual way on material obtained at the end of July, gave results similar to the foregoing. The yields in two experiments were 47 and 53 units per 10 half-nodules per hour. The nodules were somewhat smaller than those of *Phaseolus.* Material obtained in late October, however, gave no auxin, a reaction paralleling the cessation of growth at this time.

Myrica asplenifolia. One experiment with nodules (about 1 mm. in diameter) of *Myrica asplenifolia* gave for the apical halves an average of 13, and for the basal halves an average of 40, auxin units per 10 half-nodules per hour. Little significance need attach to the differences between apical and basal halves either in this or other experiments, since the bacterial tissue is not evenly divided between the two halves.

Wherever root tips were tested, only very small yields of auxin were obtained. This is in accord with the findings of Boysen-Jensen[1] that to obtain appreciable yields by diffusion from root tips, dextrose must be added to the agar.

INJECTION OF AUXIN INTO LATERAL ROOTS

As previously reported[5] the application of indole-acetic acid in lanolin paste to lateral root initials completely inhibited their elongation and caused strong swelling of the base. In an attempt to imitate more closely the conditions of continuous auxin supply within the nodule, fine glass needles containing small volumes of concentrated auxin solution were inserted into lateral roots of different ages. Pea seedlings grown on moist filter paper in light were used.

When indole-acetic or α-naphthalene-acetic acid was thus injected into a young lateral root initial not more than 0.5 mm. long, the development of the rootlet was inhibited, usually completely. Swellings of the initial itself and of the main root at the point of injection reached, in some instances, three times the normal diameter. When the acid was injected into the growing zone of developed lateral roots their elongation was stopped, and a marked swelling, in some instances almost spherical in shape, developed around the point of

* Supplied by Dr. C. D. LaRue.

injection. The artificial production of genuine nodules in this way, however, has not been achieved.

PRODUCTION OF AUXIN BY RHIZOBIA IN CULTURE

The objection was quite justifiably raised by Wilson[7] that there was no evidence that the bacteria are themselves responsible for the auxin production of the nodule. Although the data above indicate that the auxin does come from the bacterial tissue, further proof is desirable, especially from pure cultures.

Frieber[3] has shown that it is a common property of many bacteria to form indole-acetic acid from tryptophane, and Link[4] subsequently indicated that *Rhizobium phaseoli* produces auxin when grown on tryptophane-dextrose media. It was thought worth while to make a more extensive study of this point, using media without added tryptophane as more comparable to conditions within the root.

Pure cultures of *R. leguminosarum*, *R. trifolii*, and *R. phaseoli** were grown in a yeast-extract-mannite-salts medium, and samples were removed from time to time for auxin tests. After an initial period of 13 days at 28°, in which auxin production is small, the concentration of auxin was found to rise to very high levels in the culture medium. The maximum value, reached in 28 days, approximates 1 mgm. per liter, which compares favorably with the yields obtained from *Rhizopus suinus*, previously used for the preparation and isolation of indole-acetic acid. Of the three species, *R. leguminosarum* was the most active in formation of auxin, but all developed considerable quantities.

Similar results, but with a still longer lag period (about 20 days), were obtained with a medium consisting of a decoction of pea seedlings containing 1 per cent sucrose.[2] On this medium all three organisms produced auxin, but again *R. leguminosarum* was the most active. The final concentrations reached were of the same order of magnitude as those on the yeast-extract medium.

CONCLUSION

The theory that the nodule arises as a direct result of the production of auxin by *Rhizobia* within the infected root is supported by the following facts:

Rhizobia form auxin in considerable quantities in culture media the composition of which cannot differ widely from the conditions within the root.

The nodules of a variety of plants, including nonlegumes, contain relatively large amounts of auxin, and the type of auxin production apparently differs from that in normal plant tissues.

* Obtained through the courtesy of Dean Burk, of Washington.

The application to roots of pure auxins stimulates the development of lateral roots but inhibits their elongation and causes their deformation and swelling.

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STUDIES ON THE MECHANISM OF SYMBIOTIC NITROGEN FIXATION

W. W. Umbreit and P. W. Wilson

Department of Agricultural Bacteriology, University of Wisconsin, Madison, Wis., U. S. A.

About ten years ago research was initiated at the University of Wisconsin for study of the mechanism of symbiotic nitrogen fixation. In view of the many practical problems awaiting solution it may be questioned whether studies on this mechanism are entirely justified. Yet a knowledge of the biochemistry of symbiotic nitrogen fixation, especially a knowledge of the controlling factors involved in the chemical steps, has much more than simply theoretical significance. Many of its aspects are directly related to applied agriculture.

Any study of symbiotic reactions requires information with respect to the separate behavior of each symbiont. In the case of symbiotic nitrogen fixation, a continual search has been made for means by which either the bacteria or the host plant alone can fix nitrogen. It is now generally recognized that if the rhizobia are able to fix nitrogen nonsymbiotically, the conditions essential for such fixation have yet to be defined. Similarly, claims for nitrogen fixation by sterile germinating pea seeds,[4] which might be taken as evidence that the plant was the nitrogen fixing agent in the symbiosis, have not been confirmed.[2]

Of interest is the question of whether the physiology of the bacteria is the same in laboratory culture as in the root nodule. The respiratory functions of suspensions of bacteria taken directly from the nodule have been compared with the same strain of organism grown on laboratory media. In response to pH, temperature, oxygen tension, various substrates, inhibitors, and in their anaerobic metabolism, bacteria from the two sources have been shown to be virtually identical.[7]

The relation of each of the symbionts to the *effectiveness* of the nitrogen fixation process has received much attention. The fact that different strains of rhizobia are consistently able to fix differing quantities of nitrogen in association with a given host plant has been recognized for some time. These examples of "strain variation" have led to classification of a species into "good" and "poor" strains. It is becoming increasingly

evident, however, that the association of a given strain of rhizobia with closely related hosts within a bacterial plant group may result in widely differing quantities of nitrogen fixed. Since this specificity of the plant-bacterial symbiosis extends even to varieties within a species of host plant, it appears that our concepts of "good" and "poor" strains are in need of revision.[8] The factors concerned in "host-plant specificity" are a matter for further study, and clearly, from a practical point of view host-plant specificity deserves considerable attention. These results stress the importance of the host plant in determining the effectiveness of the symbosis. Other properties of the host, for example, the internal metabolism of the plant, especially its carbohydrate-nitrogen relation[5] is an important factor in regulation of the symbiosis.

Although these studies form a necessary basis, they cannot be expected to yield much information about the paths by which molecular nitrogen is converted into forms which can enter the metabolic stream of the plant. The problem may be approached by the methods of classical organic chemistry and attempts made to isolate definite chemical intermediates. [1, 3] It must be recognized, however, that many reactions other than those concerned solely with nitrogen fixation occur in the root nodule and in the plant, and these must be distinguished from reactions directly concerned with the fixation process. Successful accomplishments of this differentiation is dependent to a large extent upon the criteria employed, and since these criteria have differed with investigators, widely different and often conflicting opinions have been advanced.

Aside from the inherent difficulty of distinguishing intermediates concerned with the nitrogen fixation reaction from the many other compounds present, discovery of the chemical paths through which nitrogen fixation passes will not provide complete information with respect to the mechanism, if the term is used in its fullest sense. It appears possible that a new approach, for example, adequate definition of the characteristics of the enzyme system responsible for the fixation reaction proper might furnish information which would be ultimately of greater value for control of the process in practical agriculture than knowledge of the chemical intermediates. Some progress has been made in this direction through studies on the influence of the gaseous environment on the fixation reaction. Of special interest are the responses of the fixation system to alterations in the pN_2, pO_2 and pH_2.

Red clover plants which are inoculated with an efficient strain of *Rh. trifolii* fix nitrogen independently of the pN_2 if this exceeds approximately 0.15 atmospheres. Below this value nitrogen fixation decreases with pN_2; the *pN_2-nitrogen fixed* curve, which appears to be fairly typical of a *substrate-reaction velocity* function of enzyme reaction, has a calculated Michaelis constant of about 0.05 atm. Plants given combined nitrogen grow independently of the pN_2.[6]

Fixation of nitrogen is independent of the partial pressure of oxygen over the range 0.1 to 0.4 atm. Pressures lower than 0.1 atm. or greater than 0.4 atm. decrease fixation, but a comparison of the pO_2 function of red clover plants fixing molecular nitrogen with that of similar plants assimilating combined nitrogen shows that the two are virtually identical. It is, therefore, concluded that direct functioning of oxygen in the symbiotic nitrogen fixation process is unlikely. Proposed mechanisms involving molecular oxygen probably should be discarded. Oxygen, however, is important, in an indirect way, for the fixation reaction, especially as it influences the carbohydrate metabolism of the host plant, which is probably the basis of the view that oxygen plays a role in the process.[9]

Present evidence indicates that fixation of molecular nitrogen by red clover is inhibited by the presence of gaseous hydrogen; the inhibition is proportional to the pressure of hydrogen. There is no inhibition by argon or helium. Furthermore, the inhibiting effect of hydrogen is specific, i.e., it affects plants fixing molecular nitrogen but has little, if any, effect on similar plants receiving combined forms of nitrogen. The inhibition has been established by studies in which both *total growth*[10] and *rate of growth*[11] have been used as criteria of free nitrogen assimilation. The inhibiting effect is due to the hydrogen *per se* and cannot be attributed to an accompanying impurity. Present data indicate that the inhibition is competitive with respect to nitrogen. The inhibition is an effect upon the nitrogen fixation process rather than upon plant growth and appears to be readily reversible.[11]

Inhibition by gaseous hydrogen is of interest in that it offers a valuable tool for research by providing an agent which may enable the investigator to distinguish those reactions concerned with nitrogen fixation from those involved in other aspects of nitrogen metabolism of the plant. As the nitrogen fixing system in *Azotobacter* is not inhibited by gaseous hydrogen, evidence is furnished which suggests that a fundamental difference may exist between the two systems.

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RESPIRATION RATES OF RHIZOBIUM; THEIR ESTIMATION AND SIGNIFICANCE

FRANKLIN E. ALLISON and SAM R. HOOVER

Bureau of Chemistry and Soils, U. S. Department of Agriculture, Washington, D. C., U. S. A.

Rhizobium utilizes a large variety of substrates for respiration and growth and in these processes consumes oxygen at rates slightly lower than those of most other bacterial species. Expressed in terms of Q_{O_2} (cmm. O_2 consumed per mgm. dry weight of organisms per hour) the respiration rates of alfalfa and clover bacteria, which we have studied to the greatest extent, vary between about 5 and 40 in the presence of glucose. The Q_{O_2} of most bacteria does not exceed a value of 200, although *Azotobacter* with a Q_{O_2} as high as 5,000 represents a striking exception. Our studies, a portion of which are presented more fully elsewhere,[12] show that under reasonably standardized conditions the Q_{O_2} value of a culture of *Rhizobium* at any given time serves as a fairly good index not only of the activity of the organisms but also of the use which they are making of the carbohydrates being taken into the cell. The significance of Q_{O_2} values as we have observed them and the general bearing of these observations upon the interpretation of respiration measurements, particularly those made by the resting cell technic, are discussed in this paper.

RESPIRATION MEASUREMENTS

Respiration studies with microorganisms are usually carried out either with actively growing cultures or with so-called resting cells. In studies of the first type, organisms are grown in any convenient manner, and suitable portions (usually 2 cc.) are transferred whenever desired to microrespiration vessels and the rates of oxygen uptake measured for periods of an hour or longer. The Q_{O_2} values thus determined represent both the O_2 consumed in basal respiration and any additional O_2 required in growth. Obviously the reactions involved in respiration plus growth are many and varied. In order to simplify the problem being studied English workers developed the resting cell technic for the study of substrate oxidation apart from growth. A resting cell was defined by them as a nonproliferating cell. In obtaining such resting cells the common practice has been to grow the

organisms in a satisfactory medium, centrifuge while young and active, wash, and suspend in a nitrogen-free buffer solution with substrate. Since nitrogen is omitted from the medium, and since the respiration studies are of short duration, it has been assumed by most workers that the O_2 used represented only that required for oxidation of the added substrate. Several investigators, using various species of organisms, have, however, failed to obtain complete oxidation of added substrate. In many instances this failure was undoubtedly due to assimilation (conversion into cell material), since Clifton[5, 6] and Clifton and Logan[7] have shown that, by adding sodium azide or 2:4 dinitrophenol to cultures of *Escherichia coli* to prevent assimilation the extent of oxidation of a number of substrates could be increased from 50 to 75 per cent to near completeness. Obviously the original definition of a resting cell is not entirely satisfactory. A truly resting cell is one which is neither proliferating nor assimilating.

Our studies have been chiefly with *Rhizobium meliloti* 131 of the University of Wisconsin cultures, although *Rh. trifolii* 205 has been employed in several experiments. They were grown in shallow liquid cultures in Erlenmeyer flasks for varying periods, usually 3 days. Several experiments showed that aeration with a stream of air did not appreciably alter the metabolic characteristics of the organisms, and hence this procedure was not adopted. The bacteria were ordinarily grown at 28°C. in the basal medium[2] regularly used in this laboratory, with additions of various sugars and nitrogen sources as desired, together with adequate iron and the growth factor, coenzyme R. Respiration studies were conducted both with growing uncentrifuged organisms and with the organisms that had been centrifuged and washed once with some of the sterile culture medium. The usual Warburg technic[9] was used at a temperature of 28°C., the optimum for growth and therefore presumably for most cell activities, although this temperature does not give the maximum respiration rate, which is obtained at 37.5°C.[14] The von Fellenberg[10] method for oxidizable organic matter was usually employed in the determination of the quantity of bacteria and sugar present.

OBSERVATIONS WITH NONGROWING RHIZOBIA

When *Rh. meliloti*, grown on the aforementioned complete medium, is centrifuged, washed, and suspended in a glucose medium without added nitrogen, the Q_{O_2} usually varies between 5 and 8 depending upon the age and condition of the organisms. Very young organisms may occasionally give values as high as 10, whereas old organisms (7 to 10 days) may often give values even below 5. Q_{O_2} values of 6 to 9 are typical. We have never obtained values higher than 10 for such organisms grown on a complete synthetic medium and maintained in a glucose or sucrose medium

without nitrogen and at 28°C. Such organisms will ordinarily continue to oxidize carbohydrates for several hours without appreciable increase in cell numbers or weight and are essentially truly resting cells. If for some unusual reason, however, an increase in dry weight should occur, the organisms could not then be considered as strictly resting cells.

In the absence of substrate, centrifuged and washed rhizobia respire at an initial rate corresponding to a Q_{O_2} of 4 to 6, but this value usually drops 50 per cent or more (to 1 to 3) within a period of one or two hours, depending upon the thoroughness of washing and the supply of carbohydrate reserves in the bacteria when the values are determined. This slow respiration rate in the absence of substrate represents endogenous respiration, that is, the oxidation of cell constituents.

In the resting cell technic the method used for preventing growth is the omission of nitrogen from the medium. In our studies with *Rh. meliloti* and *Rh. trifolii* we have observed that the same result can be obtained by omitting either nitrogen or coenzyme R from the medium, provided, of course, that the organisms have been grown on a minimum content of the growth substance and have been washed thoroughly. This latter point needs to be emphasized, for, in many instances, additions of coenzyme R to centrifuged organisms have little effect because such organisms are already well supplied with the substance. This growth factor, which under favorable conditions commonly produces a two- to four-fold effect on the respiration rate during the first two hours when the organisms are in a medium containing substrate and nitrogen, fails to produce such an effect when nitrogen is omitted. This indicates that the effect of this essential substance on respiration is definitely interrelated with its effect on growth, contrary to the implications of an earlier statement;[1] we have so far been unable to stimulate respiration markedly without a subsequent simulation of growth.

OBSERVATIONS WITH GROWING RHIZOBIA

If young centrifuged bacteria are placed in a medium containing available nitrogen, carbohydrate, and coenzyme R, so that normal growth can take place, the observed Q_{O_2} is increased considerably above the resting value of about 8. In the presence of glucose, asparagin, and yeast extract, and under favorable conditions, a Q_{O_2} of about 40 (at 28°C.) may occasionally be observed. More often the value is 25 to 35. On the ordinary glucose medium containing nitrate or ammonia nitrogen and coenzyme R, values of 20 to 35 are common. Studies of organisms of various ages showed that there was a close correlation between the Q_{O_2} values and the growth rates, as evidenced by changes in dry weight of organisms per cubic centimeter of culture. The Q_{O_2} was highest at or near the period

of logarithmic growth and dropped gradually but fairly rapidly thereafter to a value of near 10 when growth had virtually stopped. As far as we

TABLE 1

RATES OF RESPIRATION OF GROWING AND NONGROWING RHIZOBIA AND *Azotobacter*

Original medium	Medium used in respiration measurements		Condition of cells	Q_{O_2}	
	Nitrogen	Substrate		Rhizobium 28°C.	*Azotobacter** 30°C.
Complete with glucose	none	glucose	chiefly nongrowing	5–10	1,000–2,000
	none	none	nongrowing	1–6	10–20
	present	glucose	growing	8–40	1,000–5,000
Yeast without glucose...	none	glucose	?	18–24†	

* Data from publications of D. Burk and H. Lineweaver.
† Data from publications of P. W. Wilson, recalculated to 28°C.

have observed, anything that increases the growth rate of rhizobia also increases the Q_{O_2}, and the reverse is probably commonly true. A Q_{O_2} value covering the range above approximately 8 therefore seems to be characteristic of growth just as lower values indicate truly resting cells. Above the point where growth starts the increase in cell weight rather closely parallels the increase in Q_{O_2}.

The Q_{O_2} values for growing and nongrowing rhizobia, given above, are summarized in table 1. Corresponding values for *Azotobacter* are included for comparison. Nearly all other bacteria have Q_{O_2} values that are intermediate between these two bacterial species.

RESTING CELL TECHNIC AS APPLIED TO RHIZOBIA

The resting cell technic has usually been applied to the study of organisms which have resting Q_{O_2} values on glucose of 20 to 50, or in the case of *Azotobacter*, near 1,000. In such cases the Q_{O_2} values in the absence of substrate are seldom more than 20 per cent as high as with substrate, and in *Azotobacter* may be even as low as 1 per cent. Furthermore, in many cases endogenous respiration has been proved to be negligible, that is, nearly eliminated in the presence of substrate. Under these conditions the method has yielded much valuable information. The conditions with rhizobia, however, are different. It was pointed out above that suspensions of rhizobia cells, prepared according to the usual resting cell technic, usually show an endogenous respiration amounting to 30 to 60 per cent of the respiration rate with substrate, and the Q_{O_2} values are low. This means that as the

reaction to be studied, namely, substrate oxidation, may be complicated to some extent at least by endogenous respiration, it is difficult to interpret the results obtained. Our repeated attempts to use the method have not proved very satisfactory; at least the results have been much less conclusive than those obtained by Burk[4] and Lineweaver[13] with *Azotobacter*, and by a large number of other workers with a variety of organisms, particularly *Es. coli* and yeast.

Wilson[14] has attempted to overcome the difficulty resulting from the low rate of respiration of organisms grown on a normal medium containing adequate carbohydrate by using young rhizobia grown on a yeast medium without other substrate. These organisms were high in nitrogen content and very deficient in carbohydrate. In the presence of glucose and in the absence of added nitrogen they gave Q_{O_2} values of 30 to 40 at 34°C. (equivalent to 18 to 24 at 28°C.). It has been our observation that such values are obtained only with growing cells. The question arises, therefore, as to whether these high-nitrogen bacteria are truly resting.

Though we have not worked with the very high nitrogen type of cell, our general observations indicate that these cells probably assimilate part of the carbohydrate. Such cells would increase in dry weight and behave essentially as do normal growing organisms. During the short periods of time used in respiration studies, cell numbers may show little or no change, but it is possible and probable that in many instances the initial processes in cell division have started.

It would seem that young rhizobia of abnormally high nitrogen content and starved for carbohydrate should show a high rate of assimilation, since almost any species of organism of this type will rapidly take up added carbohydrate until a more nearly normal carbohydrate-nitrogen ratio is attained. In Wilson's experiments only 60 per cent of the added substrate was oxidized, which indicates that assimilation was occurring. Wilson accounted for the remaining substrate by considering it to have been used in gum formation rather than converted into cell material, although he fully realized the latter possibility. Using actively growing rhizobia, we[12] repeatedly observed only a 60 per cent oxidation of added substrate and found on analysis that under our conditions the formation of gum and of other by-products was negligible, and that the unoxidized carbohydrate was converted into cell material. We are inclined to believe that the same is true of the high nitrogen cells used by Wilson.

GENERAL BEARING ON RESTING CELL TECHNIC

The tendency of various species of microorganisms that are young and are high in protein to assimilate or store carbohydrate during short experimental periods, such as those used in respiration experiments, is common.

This is shown by the studies of Cook and Stephenson,[8] Barker,[3] Giesberger,[11] and Winzler and Baumberger,[15] as well as those of Clifton and Clifton and Logan, already mentioned. Winzler and Baumberger note that "with nonproliferating cells it is not generally appreciated how great the assimilation may be in relatively short periods." In their own experiments with yeast suspensions they observed a storage of 75.5 per cent of added glucose.

The various observations with rhizobia, already discussed, as well as the experiences of other workers with the resting cell technic would seem to lead logically to the following generalizations regarding this technic: (a) A resting cell should be defined not as merely a nonproliferating cell but as a nonproliferating and nonassimilating cell, as we have emphasized above. (b) In using resting cells, therefore, the experimenter should be prepared to prove the condition of these cells by showing that the added substrate is completely oxidized or that no increase of dry weight has occurred during the experimental period. Admittedly it may be difficult to comply with these requirements in many cases, especially where the experimental period is very short. (c) There would seem, at present, to be no need to differentiate between assimilating and proliferating organisms, since from a metabolic point of view it usually makes little difference whether a substrate is used to make a given cell larger or to make the cell into two cells. Assimilation involving storage of fat or other compounds that are usually more or less inert, as far as cell metabolism is concerned, might possibly be considered as an exception. (d) The present so-called resting cell technic may be used profitably in some types of studies even with cells that are known to be actively assimilating, and even proliferating, if care is taken to interpret the data accordingly.

The foregoing remarks apply to the study of organisms in general. With regard to rhizobia in particular we can do little more than repeat that the resting cell technic is seldom satisfactory for use with truly resting cells, because of the very low metabolic rates involved. If this technic is used with assimilating or growing bacteria, the interpretation of the data may be very difficult.

SUMMARY

Respiration studies with *Rhizobium meliloti* and *Rh. trifolii* that were not assimilating carbohydrate or undergoing cell division usually gave Q_{O_2} values on glucose of 5 to 8 at 28°C., the values being highest with the younger organisms. In the absence of substrate (endogenous respiration) most of the Q_{O_2} values were 2 to 5, and some were even lower, depending largely upon the initial carbohydrate reserves in the cells. Q_{O_2} values above approximately 8 ordinarily indicated growth or assimilation; above this point the dry weight increase was closely proportional to the increase in the rate

of respiration. The maximum value observed for growing organisms on glucose was about 40.

Coenzyme R, which is essential for respiration and growth, markedly increased respiration only under conditions where a subsequent growth stimulation occurred. This indicates that there is an interrelation between its effects on the two processes.

The resting cell technic for measuring substrate oxidation was found to be rather unsatisfactory for use with truly resting rhizobia because of their low Q_{O_2} values on glucose and the small difference between values with and without substrate. This technic may, however, sometimes be used profitably with assimilating and proliferating cells, but the results obtained obviously represent both substrate oxidation and growth. It is emphasized that a resting cell should be defined not as merely a nonproliferating cell but as a nonproliferating and nonassimilating cell.

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SUR LA LYSO-RESISTANCE DU B. RADICICOLA ET SON IMPORTANCE PRATIQUE

A. Demolon et A. Dunez

Centre National de Recherches Agronomiques, Versailles, France

Les cultures pures de *B. radicicola* obtenues à partir de nodosités de légumineuses subissent fréquemment après une série de repiquages une lyse totale qui s'oppose à leur multiplication ultérieure. Ce phénomène transmissible est dû à la présence d'un bactériophage dont nous avons établi le rôle dans la fatigue des sols (soil sickness). La virulence des bactériophages et la sensibilité à leur action des divers *B. radicicola* se montre très variable.

Pour mesurer l'activité lytique d'un bactériophage, nous avons adopté la technique de d'Herelle mais en utilisant une émulsion plus riche renfermant 500 millions de germes par cmc. Au cours de passages successifs en milieu de culture liquide suivis de filtration sur bougie, on voit l'activité augmenter jusqu'à un certain maximum. Dans certains cas celui-ci ne dépasse pas 10^{-4}, on assiste alors à la production de cultures secondaires, souvent la limite observée a atteint 10^{-8} et cette valeur a pu être dépassée dans les observations de Vandecaveye et Katznelson.[6] Il convient toutefois de noter que ces mesures n'ont pas de valeur absolue; elles dépendent notamment de l'étalon adopté, c'est-à-dire de la plus ou moins grande sensibilité de la souche de *B. radicicola* utilisée.

VARIATION DANS LA SENSIBILITÉ DE DIVERSES SOUCHES DE B. RADICICOLA MEDICAGO

Dans l'expérience ci-après nous avons fait agir comparativement diverses souches de *B. radicicola* de notre collection sur une série de bactériophages provenant de luzernières fatiguées situées dans diverses régions de la France.

On voit que les différentes souches expérimentées se sont montrées très inégalement sensibles à chacun des bactériophages comme l'ont également constaté Laird,[4] Almon et Wilson,[1] Vandecaveye et Katznelson. Aucune souche n'a présenté de résistance totale. Les souches d'origine secondaire, obtenues en présence de bactériophage, manifestent toujours un accroissement de leur résistance par comparaison avec les souches primitives. Enfin signalons que les souches les plus sensibles nous ont donné un grand nombre de petites nodosités, analogues à celles décrites par Thornton,[5] réparties sur

TABLEAU 1

SENSIBILITÉ COMPARÉE À LA LYSE DE DIVERSES SOUCHES* DE *B. Radicicola medicago*

Origine du bactériophage	S et O		Bretagne		Vaucluse		Alpes Maritimes		Anglais	
	S.P.	S.S.	S.P.	S.S.	S.P.	S.S.	S.P.	S.S.	S.P.	S.S.
Seine et Oise.....	++	+	++	+	++	−	+++	−	+	−
Bretagne.....	++	+	+++	+	+++	+	+++	+	++	+
Vaucluse.....	+++	+	+++	+	+++	+	+++	+	+++	+
Alpes Maritimes..	+++	++	+++	++	+++	−	+++	++	+++	++

* Lyse complète en 24 h. sans culture secondaire +++; lyse complète en 48 h. mais donnant des cultures secondaires ++; lyse partielle en 72 h. +; aucune lyse −. S.P. = souche primitive; S.S. = souche secondaire.

l'ensemble des racines, avec une efficacité fixatrice atténuée par rapport aux souches résistantes.

COMPORTEMENT DES B. RADICICOLA SPÉCIFIQUES DES DIVERSES LÉGUMINEUSES À L'ÉGARD D'UN MÊME BACTÉRIOPHAGE

Nous avons recherché si la spécificité observée pour le *B. radicicola* des diverses légumineuses se retrouve en ce qui concerne le bactériophage. À cet effet, nous avons examiné le pouvoir lytique d'une série de bactériophages provenant de diverses légumineuses soumises à une culture continue en France et utilisés à leur *seuil* d'activité (concentration minima assurant la lyse totale) (tableau 2).

TABLEAU 2

SENSIBILITÉ COMPARÉE À LA LYSE* DE *B. radicicola* DE DIVERS GROUPES

Provenance du bactériophage	Seuil d'activité	Provenance du *B. radicicola*							
		Luzerne	Trèfles			Minette	Lupin	Pois	Haricot
			Blanc	Incarnat	Violet				
Luzerne.....	10^{-6}	+++	+++	+++	+++	+++	+++	+++	+++
Trèfle blanc....	10^{-5}	+	+++	+	+	+	0	0	0
Trèfle incarnat..	10^{-5}	+	+++	+++	+++	+	0	0	0
Lupin.....	10^{-4}	+	+	+	+	+	++	+	+
Pois.....	10^{-4}	+	0	+	+	+	0	+++	+++

* Aucune lyse 0; lyse totale +++; lyse donnant des cultures secondaires ++; lyse partielle +.

Le bactériophage de la luzerne qui présente l'activité maxima a lysé totalement à la concentration indiquée tous les *B. radicicola*. À des concentrations plus élevées, les autres bactériophages ont été plus actifs vis à vis du *B. radicicola* correspondant, mais ils ont assuré une lyse partielle de *B. radicicola* relevant de groupes différents. Il ressort donc de ces résultats que le bactériophage ne possède pas la spécificité stricte propre aux *B. radicicola* dont chaque race n'infeste qu'une légumineuse donnée.

OBTENTION DE GERMES LYSO-RÉSISTANTS

Comme nous l'avons indiqué, les germes isolés directement à partir des nodosités sont plus ou moins sensibles à l'action du bactériophage mais il est rare qu'ils résistent totalement à la lyse. Nous avons signalé plus haut que cette résistance augmente pour les cultures secondaires. Nous avons utilisé cette observation et nous avons pu obtenir une souche de *B. radicicola* medicago à la fois active au point de vue symbiotique et en pratique insensible au bactériophage en opérant comme suit: une jeune culture de *B. radicicola* a été mise pendant 48 heures au contact d'un filtrat bactériophagique à son seuil d'activité 10^{-8}. Malgré une lyse apparemment totale, il subsiste quelques individus qu'il est possible d'isoler à nouveau en ensemençant le dépôt centrifugé sur gélose et en observant les tubes pendant plusieurs jours. Après repiquage, ces germes ont servi à l'inoculation de graines de luzerne préalablement stérilisées et cultivées en milieu aseptique. À partir des nodosités prélevées vers le quarantième jour, nous avons obtenu une souche de résistance accrue qui de nouveau a été soumise à l'action d'un lysat bactériophagique plus concentré que précedemment. Après 6 opérations analogues avec passages par la plante, la résistance de la souche à la lyse était pratiquement totale; par ailleurs elle présentait à la fois un pouvoir infectant élevé à l'égard de la plante et une activité fixatrice satisfaisante, même dans un milieu contaminé par le bactériophage. Au champ, les conditions ne semblent pas permettre la production de races résistantes à une vitesse suffisante pour éviter la dépression de l'activité symbiotique qu'on constate dans les sols fatigués.

UTILISATION DES SOUCHES LYSO-RÉSISTANTES

Une souche résistante obtenue comme il vient d'être indiqué a servi à inoculer des graines qui ont été ensemencées sur une terre fatiguée où la luzerne, après trois années de culture, était en voie de dépérissement (tableau 3). Le sol fut retourné par un simple labour. Dans les parcelles témoins et dans celles où la graine avait été inoculée avec germes sensibles, la végétation a été médiocre et la fatigue a réapparu malgré l'atténuation du bactériophage par la sécheresse. L'utilisation de la souche résistante a assuré une végétation vigoureuse qui s'est parfaitement maintenue en seconde et en

troisième année. L'examen des racines montrait dans un cas la présence de nodosités nombreuses et bien développées avec bactéroïdes sains, et dans le second cas la présence de rares nodosités en surface avec dégénérescence granuleuse des bactéroïdes.

TABLEAU 3

UTILISATION DES GERMES LYSO-RÉSISTANTS DANS L'INOCULATION DE LA LUZERNE

Rendements en kgm. matière sèche par hectare

	Ière année 1934	2ème année 1935	3ème année 1936	Nouveau semis avec graines inoculées (germe très résistant) 1937	5ème année 1939
Témoin.....	5.700	6.900	2.500	*3.600*	*4.500*
Inoculation avec germe moyennement résistant.	6.400	8.240	4.100	*6.700*	*10.300*
Azote total fixé par la récolte avec inoculation (kg. par ha).....	254	275	139	*236*	*382*

Cette expérience répétée ailleurs, vient confirmer nos conclusions antérieures[3] sur le rôle du bactériophage dans la fatigue des sols portant des légumineuses. Elle montre l'intérêt pratique qui s'attache à l'utilisation de germes à la fois actifs et lyso-résistants dans l'inoculation des graines. On assure ainsi à la plante le bénéfice d'une symbiose susceptible de se maintenir même en milieu contaminé. Il devient alors possible de conserver la luzerne plus longtemps ou de la faire revenir plus fréquemment sur le même sol.

It is possible to obtain strains of *B. radicicola* both resistant to lysis by the phage and highly efficient with respect to nitrogen fixation. Field experiments with alfalfa-sick soils indicate that use of such strains for inoculation of seeds offers considerable advantage.

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BACTERIOPHAGE AND THE LEGUME BACTERIA

H. KATZNELSON

New Jersey Agricultural Experiment Station, New Brunswick, N. J., U. S. A.

Rhizobium-phage has been isolated by many investigators, from Gerretsen, et al.[9] in 1923 to Demolon and Dunez[6] in 1936. The lytic agent has been obtained from soil planted to legumes;[3, 5, 6, 22] from nodules, roots, and stems of leguminous plants;[5, 6, 8, 9, 10, 11, 13, 22] and from stock cultures of the nodule bacteria.[13] Not all the soils, nodules, stems, or roots tested have yielded bacteriophage. There is some disagreement as to the phage incidence in nodules of different ages. Laird[13] maintains that the lytic agent can be isolated from distinctly young nodules but only with difficulty, if at all, from old nodules, whereas Demolon and Dunez[5] claim they have never found phage in young nodules.

Phages for a variety of legume bacteria (producing nodules on clover, alfalfa, vetch, peas, beans, etc.) have been isolated and have been shown to be nonspecific. In addition *Rhizobium* is heterogeneous with respect to phage in that it comprises strains some of which are resistant, others susceptible, but no difference could be demonstrated[1, 10, 13] between susceptible and resistant strains in regard to their effectiveness (in producing nodules and in symbiotic nitrogen-fixation).

Rhizobium-phage is most active at pH 7.5-8 but also brings about lysis at pH 6.[13] Its activity is greatest under conditions which reduce slime production by the nodule organism to a minimum and which are optimum for the development of the homologous organisms. Demolon and Dunez[5] have found that soil does not fix bacteriophage appreciably and that desiccation and exposure to the sun of soils containing the lytic agent result in its destruction.

During the last few years Demolon and Dunez[5, 6, 7] have presented evidence pointing to the lytic principle as being indirectly responsible for "fatigue" of alfalfa-bearing soils in France. The lytic principle in destroying the *Rhizobia* in soils and nodules interferes with normal symbiosis and forces the plant to grow as a nonlegume. Nevertheless, other investigators[3, 22] after isolating *Rhizobium-phage* from many soils came to no such definite conclusions, although their data by no means obviate the possibility. It is not the purpose of this paper to discuss the pros and cons of this question.

Before the question can be considered solved, however, more exhaustive work is required regarding the effect of resistant strains in soils and their ability to produce nodules in the presence of phage, as has been shown to be the case in sand cultures;[1, 13] absorption of phage by soils and plants; persistence of the lytic agent in different soils under varying conditions; the effect of the soil microflora on the phage; and the factors of the environment (reaction, moisture, nutrients, antagonistic effects) affecting the survival of *Rhizobia* in soil.

The present study deals with the capacity of various substances (soils, organic residues, carbon, bentonite, etc.) and biological systems to absorb *Rhizobium-phage*. This may have practical significance with respect to phage dissemination and activity in soil.

That bacteriophage is readily absorbed by inorganic and organic colloidal complexes is well known. Recently considerable work has been done on the absorption of phage by homologous susceptible and resistant living and dead bacteria and by unrelated organisms.[2, 12, 14, 15, 17, 18, 19, 20, 21] It has been found that phage is absorbed by living or dead susceptible bacteria but infrequently by resistant forms.[2, 20] Unrelated bacteria also occasionally absorb a particular lytic agent.[18, 19]

EXPERIMENTAL

A method commonly used in such studies is the plaque-count technic. A bacteriophage filtrate may be diluted and plated in much the same way as bacterial suspensions are, with the exception that in the plaque method a suspension of homologous susceptible bacteria must be added to every plate. After 48 hours' incubation, clear areas (plaques) are produced in the confluent bacterial growth. The plaques are considered to originate from phage corpuscles fixed in place by the solidified agar and lysing the adjacent susceptible organisms. This method is obviously well adapted to determining the concentration of phage particles in any given filtrate and is, in fact, commonly used. The following procedure was employed in this study. A suspension of a young agar culture (18-24 hours) was made up with medium 5 [a yeast water, mannitol-free medium of Laird[13]] to a constant volume (9 cc.) and turbidity. To this suspension was added 1 cc. of a diluted phage filtrate, and the mixture was shaken at frequent intervals and kept in the refrigerator (to prevent multiplication of the bacteria) for 5 hours. As a control, 9 cc. of the fluid medium plus 1 cc. of the same dilution of phage was maintained along with the culture. The cultures and controls were each filtered through individual sterile Berkfield candles and the filtrates dispensed in suitable dilutions in petri plates to which 1 cc. of a uniform suspension of a susceptible strain had been added; 10 cc. of a melted and cooled agar medium [medium 4 of Laird[13]] was then poured

into each of the plates, and the whole was thoroughly rotated. The plates were incubated for 48 hours at 28° C. and the plaques counted.

The technic described was used throughout. The choice of bacterium-phage contact time was arbitrary, as was the turbidity standard. It has been found that an increase in the number of suspended cells results in an increased rate of absorption and hence in a shorter time of contact.[18] Comparable results may be readily obtained, however, by the means employed.

Rhizobia were used in the first series of experiments. Strains A2 and A1, susceptible and resistant respectively, were the same cultures studied in a previous investigation,[22] the remainder comprised different members of the cross-inoculation groups.* Of these, *Lupine* 527 and *Medicago sativa* 620 were slightly susceptible to the alfalfa-phage isolated by Vandecaveye and Katznelson[22] and used in all the following experiments. The results are presented below:

Rhizobium strain	Alfalfa A2	Alfalfa A1	Sweet clover 620	Sweet clover 617	Sweet clover 142	Clover 662
Number of plaques $\times 10^{-6}$ (average of 4 plates)....	5	286	10	17	25	290

Rhizobium strain	Crimson clover 2851	Austrian winter pea 524	Vetch 420	Austrian winter pea 2828	Alaska pea 421	Garden bean 162
Number of plaques $\times 10^{-6}$ (average of 4 plates)....	272	268	266	281	279	291

Rhizobium strain	Garden bean 342	Lupine 527	Soybean 258	Black locust 715	Astragalus rubyi D	Control
Number of plaques $\times 10^{-6}$ (average of 4 plates)....	259	276	258	265	261	270

It is evident that alfalfa-phage was absorbed only by strains belonging to the same cross-inoculation group. Even within this group, an irregularity occurred, in that strain A1, the resistant organism, did not absorb the lytic

* Obtained through the courtesy of L. T. Leonard of the U. S. Department of Agriculture.

agent. Nevertheless, with the possible exception of strain 620, the sweet clover organisms were not susceptible, yet they also absorbed the phage. Such discrepancies have been observed by other investigators.[2, 19] Congress[4] found, too, that a susceptible strain of *B. coli* did not absorb its homologus bacteriophage, as was the case with the slightly susceptible Lupine strain 527. Specific bacteriophages have been used to identify such organisms as dysentery bacteria.[16] Rakieten and Rakieten[18] intimated that phage absorption may be used to detect antigenic relationships among organisms serologically unrelated. The foregoing results suggest the possibility that phage absorption may be utilized to distinguish between members of different cross-inoculation groups. The technic is so simple and rapid that it would greatly facilitate the characterization of *Rhizobia* as belonging to a particular group of these legume bacteria. Of course, much more extensive work is necessary, especially with phages active against strains other than those belonging to the alfalfa group, before any significance may be attached to this possibility.

Several other organisms were tested in a similar manner with the following results. No absorption could be demonstrated.

Organism	Soil spore former (*B. simplex*) type	*Ps. fluorescens*	*Az. chroococcum*	Control
Numbers of plaques $\times 10^{-6}$...	206	190	205	220

A number of typical soil fungi (*Rhizopus, Trichoderma, Penicillium, Aspergillus,* and *Fusarium* species) were next used. They were inoculated into 15 cc. of a suitable phage dilution containing a small amount of dextrose. After incubation for 20 days, the fungus cultures were filtered through paper then through Berkfeld candles, and the filtrates were tested for plaques. The following results were obtained:

Fungus	*Rhizopus* sp.	*Trichoderma* sp.	*Penicillium* sp.	*Fusarium* sp.	*Aspergillus* sp.	*Control*
Number of plaques $\times 10^{-6}$..	0	275	81	286	266	260

Phage inactivation has evidently occurred with *Penicillium* and, more especially, with *Rhizopus*. The reaction of the filtrates was above neutral-

ity. Mats of these fungi were prepared by inoculating these organisms into the fluid medium used in the preceding studies and containing a small amount of sugar. These mats were placed with a phage dilution in the refrigerator for 20 days. No absorption could be demonstrated. It is suggested either that the fungi were capable of breaking down and utilizing the phage or that during their growth they produced substances which inactivated the lytic agent. Further experiments with this phase of the work are in progress.

Finally, studies were made with a variety of substances, both complex and simple. The quantities of these and of phage used and the results obtained are presented below. Again the time of contact was 5 hours, the samples being kept in the refrigerator and stirred frequently.

Materials used	Amounts	Quantity of phage dilution used	Number of plaques $\times 10^{-6}$ (Average of 4 plates)	pH of filtrate
	gm.	*cc.*		
Soil 5B*	20	20	4	6.4
Palouse silt loam	20	20	3	6.6
Activated carbon	1	15	0	7.2
Bentonite	0.5	20	0	7.6
Ground alfalfa	1	15	152	7.2
Ground wheat straw	1	15	76	7.2
Lowmoor peat	2	20	0	5.6
Highmoor peat	1	20	0	5.6
Control			145	7.5

* Manured and limed Sassafras soil.

There is evidence of marked absorption of bacteriophage by all the materials except straw, which absorbed the phage to some extent, and alfalfa, which did not absorb the lytic agent. The peats used may have inactivated the phage by virtue of their low pH. A suitable phage dilution was accordingly brought down to a pH of 5.5 and maintained for 5 hours in the refrigerator. Subsequent tests showed only a slight decrease in plaque count, hence it is reasonable to infer that some absorption of phage by the peats did occur. Exposure to lower pH values and to pH 5.5 for longer periods resulted in the inactivation of the principle. The results with the two soils are at variance with those obtained by Demolon and Dunez[5] who could not demonstrate any appreciable decrease in activity of bacteriophage after contact with soil for 24-48 hours.

DISCUSSION AND SUMMARY

The possible role of phage in destroying legume bacteria in soils and nodules and thus interfering with symbiosis warrants further, more meticulous study of this lytic principle. Lines along which such investigations should be carried out have been suggested earlier. In the present work phage absorption by various substances and by certain microorganisms has been examined as such substances and such biological systems are present in soils and may therefore influence phage activity in these complex, heterogeneous substrates. It is evident from the results obtained that marked absorption by different systems did occur. *Rhizobia,* belonging to the same cross-inoculation group for a strain of which the phage used was active, absorbed this lytic agent. It was suggested, therefore, that the method might be useful in identifying strains of *Rhizobia* with a particular legume group. Of the remaining organisms tested (*Az. chroococcum, Ps. fluorescens, B. simplex* type, and various fungi), only *Rhizopus* and to some extent *Penicillium* affected the lytic agent, possibly by utilizing the agent as a nutrient or by inhibiting or inactivating it through the production of toxic substances during growth. Two soils, activated carbon, bentonite, lowmoor and highmoor peats, and, to some extent, ground wheat straw absorbed or inactivated *Rhizobium-phage.* Such extensive absorption warrants careful consideration before conclusions are drawn concerning the influence of phage on *Rhizobia* in nature.

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SYMBIOTIC PROMISCUITY IN THE LEGUMINOSAE

J. K. WILSON

Cornell University, Ithaca, N. Y., U. S. A.

NUMEROUS reports concerning the specificity of the relationship existing between the leguminous plants and their associated organisms have appeared in scientific journals. These reports emphasize the fact that not all species of the Leguminosae symbiose with the same type or strain of Rhizobium. This difference in apparent specificity led to the projection of plant-bacteria groups in which certain plants and certain strains of the rhizobia were promiscuous. In one plant group more than 40 species were included, and in others a single species was listed. This made it appear that a specific plant and a specific type or strain of the rhizobia must come in contact with each other before symbiosis could occur and that a certain strain was limited to symbiosing with a definite species. This point of view might have developed because of the particular strain that was encountered, because of the methods employed in making the tests, because of the limited scope of the tests, or because of other modifying conditions.

Wilson[10] presented evidence that the boundaries surrounding many legumes as related to specificity were not so rigid as had been supposed. Such a conclusion was possible because of the scope of his observations. It was noted that many species which had been tentatively assigned to an independent group would symbiose with strains of the rhizobia that had been isolated from members of other plant-bacteria groups.* It was also observed that the strain of the organism isolated from the plants assigned to the independent groups would produce nodules on members of other plant-bacteria groups. It was evident, therefore, that promiscuity was widespread between the species of plants and the strains of Rhizobium. It existed irrespective of the plant-bacteria groups that have been proposed. The present report presents more evidence concerning the extent of this symbiotic promiscuity in the Leguminosae and its relation to self- or cross-pollination.

METHODS

The requirements for the study were that good conditions for the growth of the plants should be provided and that the species and the pure culture of

* A list of the proposed plant-bacteria groups is given by Fred, et al.[2]

the organism should be housed in contact with each other during the growing period to the exclusion, at least, of all other strains of the microsymbiont. The details of such a method are found in the report by Wilson.[10] In brief, the method consists in growing the plants in closed glass containers on sterile sand that has been inoculated with the desired strain of the bacteria. The sand was wetted with a solution containing 0.05 per cent each of KH_2PO_4 and saccharose. Before being planted, hard-seeded legumes were treated with concentrated sulfuric acid and washed in water. All seeds were immersed in a solution of chlorinated lime, just before being planted. The plants were grown for about 30 days. The morphology of the strains was observed after staining by the method proposed by Gray as modified by Hofer and Wilson.[3]

PRESENTATION OF DATA

In work dealing with symbiosis it is difficult to determine the extent to which tests should be made in order to obtain an accurate picture of the extent of promiscuity between the plant and the various strains of the rhizobia. It seems impractical, for one reason or another, to include representatives of each genus of the Leguminosae, yet the more extensive the plant tests are, the clearer the picture becomes. On the other hand, it is probably just as important to employ as many strains of the rhizobia as can be handled conveniently. Since at least 16 plant-bacteria groups have been projected, and each is supposed to be represented at least by one strain of the organism carrying certain peculiar characters that make it specific for the one group or one plant, it would seem logical that no less than one culture from each group should be employed.

Symbiotic promiscuity of strains of the rhizobia as related to various species of legumes. In a test to determine the symbiotic promiscuity of strains of the rhizobia as related to various species of legumes, 15 tribes embodying 163 species were employed. The lowest number of species in any tribe is 1 and the highest is 51. The strains of the rhizobia studied represent one or more isolations from each plant-bacteria group and total 32. The only species among the 163 which failed to symbiose with any one of the strains was *Cicer arietinum*. This species did symbiose, however, with a strain that was isolated from Cicer. Nine species symbiosed with the strain from *Spartium scoparium*, and thirteen with the strain from *Onobrychis viciaefolia*. These two species were listed by Maassen and Müller[5] as separate plant-bacteria groups with a specific organism for each group. The strain from *Albizzia julibrissin*, a species that has not been assigned to any plant bacteria group, symbiosed with 94 species, whereas the one from *Stizolobium deeringianum*, a species that was placed in the cowpea plant-bacteria group by Tracy and Coe[6] and in a separate group by Walker[8] symbiosed with 105 species,

or about two-thirds of those employed. The strain from the cowpea (*Vigna sinensis*) symbiosed with 76 of the 163 species. Of importance also is the fact that the strain from *Dalea alopecuroides*, a plant listed by Whiting, Fred, and Helz[9] as being specific for one strain of the organism, symbiosed with 74 species. The strains from *Amorpha fruticosa, Amphicarpa monoica, Caragana frutescens, Glycene max, Robinia pseudo-acacia,* and *Strophostyles helvola,* species developed as separate groups by one or more investigators, symbiosed with 25, 31, 77, 66, 28, and 32 species respectively. Also strains from certain plants that have not been assigned to any plant-bacteria group were found to be promiscuous. Thus the strain from *Apios tuberosa* symbiosed with 36 species, representing 6 groups; the strain from *Laburnum vulgare* symbiosed with 28 species, representing 5 groups; the strain from *Sesbania macrocarpa* symbiosed with 85 species, representing 11 groups; and the strain from *Swainsonia coronillaefolia* symbiosed with 76 species, representing 10 groups. In each of these instances the strain also symbiosed with species that have not been proposed for any group. In addition, other similar promiscuous relations were studied. The relations of each of these strains to each species are described in a paper by Wilson.[10] In all these cases the boundaries surrounding the species as related to the microsymbiont were so irregular that it was impossible to arrange any plant-bacteria group that would provide a satisfactory explanation for the data.

Since *Cicer arietinum* did not symbiose with any strain of the Rhizobium except for one isolated from Cicer, this strain was employed in tests to ascertain whether it was specific for this one plant. For this purpose, plants were grown in the closed containers on the sterilized substrate that had been inoculated with the strain. The plants were grown in the greenhouse, and their roots were examined after about 30 days. The results showed that of 30 species, 3—*Amorpha fruticosa, Robinia pseudo-acacia,* and *Oxytropis lambertii*—symbiosed with the strain from Cicer. It appeared perfectly adapted to the last species but less so to the other two. It seems, therefore, that this strain is not specific for Cicer and may be found associating with other species of plants.

Promiscuity of Amorpha fruticosa. If promiscuity among the strains of the rhizobia and the species of legumes is as widespread as the foregoing results indicate, it should be suspected that a large number of isolations of the organism from a certain species of plants known to be promiscuous would exhibit a wide range of symbiotic relations. One or more of the strains from such a source might symbiose with species representing several plant-bacteria groups. Such strains may or may not be thoroughly adapted to various species. As an example, it is known[10] that a strain from *Trifolium pratense,* a species that was placed in the clover plant-bacteria group, will symbiose, as indicated by the presence of nodules, with *Lespedeza striata* about as well as

will the strain from *L. striata*, a plant included in the cowpea plant-bacteria group, whereas the same strain from *Trifolium pratense* will symbiose with only about 40 per cent of the plantlets of *Trifolium pannonicum*, a plant that is usually placed in the group with *T. pratense*.

In order to make a test of this nature as rigid as possible, strains of the organism were isolated from a species of plant giving every evidence that it is promiscuous. Such a plant is *Amorpha fruticosa*. It symbiosed with strains isolated from several plant-bacteria groups, and in addition it is the only representative of one of the groups. The sources of the strains from this plant were samples of soil from widely separated points on the globe. The location from which the samples came are shown in Table 1. Many of these soils were supporting species of legumes seldom encountered. Such samples might contain a high population of a strain well adapted to the particular species growing on the soil and thus make it possible for Amorpha when grown on this soil to come in contact with the particular strain. By such a procedure a wide variety of strains might be obtained. Plants that are promiscuous apparently exercise no preference for a particular strain. They may symbiose with two or more strains simultaneously whether the strains are perfectly adapted to the plant or not, for *Phaseolus coccineus* was observed by Wilson[10] to symbiose with as many as four strains at once. If the four strains were replaced by four other strains with which this plant was known to be promiscuous it would symbiose simultaneously with these also. The soil, therefore, as obtained from the widely separated points, was used as an inoculant for a sterile sandy soil contained in flasks on which plantlets from sterilized seed of *A. fruticosa* were grown. After sufficient time the roots were examined for nodules, and isolations were made from this source. Transfers were made from colonies on plates to slopes, and after being tested for purity, certain ones were used to inoculate sterile soil on which Amorpha was seeded again. This was done in order to test the power of each isolation to symbiose with this plant. The positive strains were then employed to reinoculate sterile sand on which seeds of plants representing several plant-bacteria groups were sown. The containers in which the plantlets grew were kept in the greenhouse from March 7 to April 5, 1939, after which the plants were examined for nodules. This provided a method of determining the symbiotic promiscuity. The data are shown in Table 1.

In order to present the data so that comparisons could be made, four degrees of adaptation of the strains to the various species of plants were noted; namely, excellent, good, fair, and poor. It is not certain that the promiscuous relations evident in Table 1 would appear in exactly the same order if another test of the same nature, employing the same strains, were conducted, for it would be difficult to duplicate the same conditions of light, heat, and variations in seedlings, all of which might influence the occurrence of nodules.

TABLE 1

CHARACTER OF PROMISCUOUS RELATIONS BETWEEN STRAINS OF THE RHIZOBIA FROM *Amorpha fruticosa* AND CERTAIN SPECIES OF LEGUMES*

Source of soil from which strains were isolated	Species of legumes in tests of promiscuity													
	Albizzia julibrissin	*Amorpha fruticosa*	*Astragalus mexussi*	*Cajanus indicus*	*Centrosema virginiana*	*Crotalaria sagittalis*	*Desmodium canadense*	*Lespedeza hirta*	*Medicago sativa*	*Phaseolus coccineus*	*Robinia Pseudo-Acacia*	*Sesbania macrocarpa*	*Trifolium pratense*	*Vicia villosa*
Belcher Islands, Arctic, Circ.		G							P	P				P
Bristol, Connecticut		F		E	G		F							
Charleston, S. C.		G								F				E
Cleveland, Ohio		E							E			P		
Ft. Collins, Col. (1)		F								G		E		
Ft. Collins, Col. (4)	E	G								F	P	E		
Ft. Collins, Col. (5)		G			P	E				E	E	E		
Ft. Collins, Col. (6)	P	E				E			F		P	E		
Geneva, Ohio (1)		G		F						P			E	F
Geneva, Ohio (2)		G				P	G				P		E	E
Ithaca, New York		G									E			
Iowa Falls, Iowa		P								P			E	
Lincoln, Nebraska (1)		E							P	P	F		E	
Lincoln, Nebraska (3)		F				P	G				P		E	F
Madison, Wisconsin (2)		G			F	F	G	E		E	P			F
Milano, Texas		G				P	E				F		E	F
Monterey, California (1)		E			P				F			F	E	
Monterey, California (2)				P						P	P			G
Nile River Val. Egypt		F										P	E	
North Platte, Neb. (1)	G	G										E		
North Platte, Neb. (4)		G	G		F	E	P					E		
Palestine (1)		P							G					
Palestine (2)										P				
Palestine (3)												P		
Plainfield, Wis.		G										G		
Portersville, Cal.		E											E	
Riverside, Cal.		F							E	E		F		P
San Joaquin Val., Cal.		E			F				G	P				
Sequoia Nat. Park, Cal. (1)		P				E							E	
Sequoia Nat. Park, Cal. (2)		G				E					P		E	
Tonopah, Nev.										F		G		
Tucson, Arizona (1)	E	F				E				E	P	G		
Tucson, Arizona (3)		G			G	G				E	P			
Urbana, Ill.		F							F	P			F	
Wake Forest, N. C.		G							F				E	
Winooskie River Valley, Vt.		G							F				G	
White Horse Pass, Nev.	G	P				E								
	5	33	1	3	7	12	6	1	11	17	13	14	14	9

* E—Excellently Nodulated
G—Good Nodulation
F—Fair Nodulation
P—Poor Nodulation

Yet, it is evident that promiscuity exists and that virtually no relation exists between the findings and any grouping of plants that has ever been suggested.

If a relationship exists, even approximating anything definite, the results obtained with the 46 strains from *Amorpha fruticosa* and the plants representing several plant-bacteria groups, such as alfalfa, clover, or cowpea, should make it apparent. No such relationship, however, is indicated. On the contrary, it is seen that many of the strains studied were better adapted to other species of plants than they were to the one from which they were isolated, or at least they symbiosed excellently with plants that represent several of the projected plant-bacteria groups. One strain the source of which was a soil from Fort Collins, Colorado, appeared excellently adapted to *Crotalaria sagittalis, Phaseolus coccineus, Robinia pseudo-acacia,* and *Sesbania macrocarpa.* Another strain the source of which was a soil from the Nile River Valley in Egypt appeared excellently adapted to *Trifolium pratense.* Still another strain from a soil from Riverside, California, appeared perfectly adapted to *Medicago sativa* and to *Phaseolus coccineus.* The strain from the soil from Milano, Texas, appeared perfectly adapted to *Desmodium canadense* and to *Trifolium pratense.* Further, a strain from the soil from Geneva, Ohio, appeared perfectly adapted to *Trifolium pratense* and to *Vicia villosa* and produced good nodulation on *D. canadense.* It is unnecessary to refer to each instance where promiscuity is evident and to the various degrees of adaptation. A study of the data in table 1 will reveal that promiscuity is widespread.

It is indicated also that certain strains from *Amorpha fruticosa* appeared better adapted to this plant than to others; 4 strains being poorly adapted, 8 fairly well adapted, 17 showing good adaptation, and 8 showing excellent adaptation. Those strains that were poorly adapted to *A. fruticosa* were well adapted to some other species, and only two instances were found in which strains from *A. fruticosa* failed to symbiose with other species. The source of one strain was the soil from Washington, Pennsylvania. This strain was excellently adapted to Amorpha. The other was from Hudson, Wisconsin, and was only fairly well adapted to Amorpha. Only four strains were so poorly adapted that they failed in this test to symbiose with Amorpha.

It should be emphasized also that 13 of the 46 strains from Amorpha appeared excellently adapted to *Trifolium pratense*; 9 to *Sesbania macrocarpa*; 7 to *Crotalaria sagittalis*; 6 to *Phaseolus coccineus*; 2 to *Albizzia julibrissin, Medicago sativa, Robinia pseudo-acacia,* and *Vicia villosa*; and 9 to *Amorpha* itself. Such data show that strains from *Amorpha* exhibit a wide range of symbiotic relations, being well adapted to species of legumes representing four or more plant-bacteria groups.

Dissimilarity of Strains from Amorpha fruticosa. Additional evidence was desired to show that the strains from *A. fruticosa* were dissimilar. To obtain

such evidence the rate of growth of giant colonies of each strain on three media was ascertained. About 10 ml. of each medium was poured into a series of petri dishes and allowed to harden. Seven giant colonies were started in each dish by making transfers from the actively growing strains to the surface of the agar medium by means of a transfer loop 1 mm. in diameter. These colonies at first appeared unusually similar, covering about 1 sq. mm. Such colonies were further developed by incubation at room temperature for 4 days. After this period the area covered by each colony was measured and the mean calculated. The readings to the nearest square millimeter are found in table 2.

If this method of showing variations in cultures is reliable, the area covered by each colony indicates extreme dissimilarity of the isolations. The strain from Hudson River Valley, N. Y., on Medium I covered 137 sq. mm., whereas one of those from Palestine covered only 6. Each of 7 cultures covered more than 100 sq. mm., each of 22 covered more than 50 sq. mm., and 4 covered less than 10. On Medium II the strain from the Hudson River Valley, New York, covered 177 sq. mm., whereas one from Ithaca, N. Y., covered only 5 sq. mm. Each of 8 cultures covered more than 100 sq. mm., each of 18 covered more than 50 sq. mm., and 5 covered less than 10 sq. mm. When the concentration of peptone was increased five times, the area covered by the colonies was drastically reduced. Not a single giant colony of any strain covered more than 28 sq. mm., and each of 30 covered less than 10 sq. mm.

Evidence supporting promiscuity of plants as a heritable character. It is apparent from the findings that certain plants are more restricted than others in their relations with strains of the rhizobia. Species such as *Albezzia julibrissin* and *Lespedeza striata* exhibit this restricted relation. Such data are in agreement with those presented by Wilson,[10] which showed that *Ulex europaens, Psoralea onobrychis,* and *Ornithopus sativus* symbiosed with only 1 of the 32 strains employed, although each strain from these three plants symbiosed with several species.

Other species were observed by Wilson[10] to symbiose with 2 of the 32 strains, others with 3 of the strains, and still others with 4, and so on until some species were observed to symbiose—some better than others—with 30 of the 32 strains. This may indicate that the plant is the dominant member in the association, and this indication is substantiated further when it is realized that species of a single genus show wide differences in their symbiobility with various strains. As an illustration, *Trifolium pannonicum* symbiosed with 3 of the 32 strains, *T. pratense* symbiosed with 16 of the strains, and 6 other species lie between these extremes. Even wider differences were found with species of Crotalaria which have been assigned to the cowpea group. The symbiobility of 19 species of this genus was determined, employ-

TABLE 2

DISSIMILARITY OF STRAINS FROM *Amorpha fruticosa* AS ASCERTAINED FROM GROWTH ON MEDIA

Source of strain	Area covered by one colony in sq. mm.		
	Medium*		
	I	II	III
Balston Spa, N. Y.	15	17	8
Belcher Islands, Arctic Circle	133	165	3
Bristol, Conn.	33	24	28
Charleston, S. C.	78	117	2
Cleveland, Ohio	26	5	9
Ft. Collins, Col. (1)	95	113	5
Ft. Collins, Col. (4)	124	20	4
Ft. Collins, Col. (5)	117	96	6
Ft. Collins, Col. (6)	123	113	8
Geneva, Ohio (1)	74	74	3
Geneva, Ohio (2)	77	79	5
Hudson River Valley, N. Y.	137	177	9
Ithaca, N. Y.	7	5	5
Iowa Falls, Iowa	79	97	4
Lincoln, Neb. (1)	49	68	1
Lincoln, Neb. (3)	28	29	3
Madison, Wis. (2)	88	78	3
Milano, Texas	115	87	8
Monterey, Cal. (1)	80	38	3
Monterey, Cal. (2)	92	40	28
Nile River Valley, Egypt (1)	120	143	3
Nile River Valley, Egypt (2)	26	...	19
North Platte, Neb. (1)	22	26	12
North Platte, Neb. (4)	66	43	5
Palestine (1)	6	10	10
Palestine (2)	13	13	12
Palestine (3)	22	30	11
Philippine Islands (1)	28	106	11
Philippine Islands (2)	7	5	13
Plainfield, Wis. (2)	18	15	20
Portersville, Cal.	61	7	3
Riverside, Cal.	13	28	11
San Joaquin Valley, Calif.	69	51	9
Sequoia Nat. Park, Cal. (2)	82	154	2
Tonopah, Nev.	13	20	2
Tucson, Ariz. (1)	20	27	5
Tucson, Ariz. (3)	58	33	3
Urbana, Ill.	31	20	1
Wake Forest, N. C.	65	95	8
Winooskie River Valley, Vt.	65	11	6
White Horse Pass, Nev.	43	64	8

* Media I. Saccharose—10 gr.
$MgSO_4$, KH_2PO_4, NaCl—0.2 gr. of each
$CaSO_4$—0.1 gr.
$CaCO_3$—5.0 gr.
Yeast extract—2.0 gr. water, 1 l.
II. 2 gms. peptone instead of yeast
III. 10 gms. peptone instead of yeast

ing 32 strains of rhizobia. *C. grantiana* appeared to nodulate perfectly with the strains from *Albezzia julibrissin, Apios tuberosa, Crotalaria spectabilis, Glycene max, Lespedeza striata, Stizolobium deeingianum,* and *Vicia villosa,* and less perfectly with the others, in some cases only one plant out of 15 or 20 bearing nodules. *C. Sagittalis* appeared to nodulate perfectly with 13 of the strains representing several plant-bacteria groups, and *C. polysperma* appeared to nodulate perfectly only with the strain from *Lupinus perennis.* Data like these, indicating a wide and restricted degree of promiscuousness, suggest that each species possesses its own selective mechanism for certain strains and that this mechanism functions independently for each plant.

When two or more species of plants were observed to symbiose with an equal number of strains of the total employed, each species did not necessarily symbiose with the same strains. This has been taken to indicate that although different species within a genus and even varieties with a species bear different relations to the various strains they may still occupy a somewhat similar position as regards their ability to symbiose with a certain percentage of the strains. Since these differences exist within a genus and even among varieties, the suggestion can be made that a major portion of the promiscuity exists within the plant itself. If this is correct then it appears that variations in promiscuity originate primarily within the plant. The mechanism by which this is accomplished is not altogether clear. Variations in numerous characters of plants are known to occur through cross-fertilization. Varieties of plants which resist certain diseases to a surprising degree have been developed by geneticists through controlled pollination. Various color characters and increased yields of fruit have been effected also by genetecists through cross-fertilization. It appears possible that this is the mechanism through which promiscuity originates or disappears. Evidence that promiscuity is associated with the degree of cross-pollination was presented by Wilson.[10] He found a striking correlation between the degree of self- or cross-pollination of legumes and their promiscuity. Species that appear to be exclusively self-pollinating do not symbiose with any known strain of the rhizobia, or if some species that are self-pollinating do bear nodules, they possess few if any promiscuous characters and symbiose with a limited number of strains of the rhizobia. On the other hand, plants that are obligatorily cross-pollinated, exhibit a high degree of promiscuity. The value of such a relation in studying promiscuousness is difficult to estimate. The relation might explain why one species will symbiose with only 1 or 2 strains of a group of 32, whereas another species of the same genus will symbiose with 29 of the same strains. It might even explain why one worker reports certain findings when employing seeds and strains of the rhizobia from one source, whereas another worker may report different findings when employing seeds and strains of the rhizobia from the same or from a different source. The

explanation might lie in the fact that the plantlets from the seed from the different sources might have had their characters for promiscuity modified by cross-pollination of the parent plant or that promiscuity existed in the plants from which the strains were isolated, with the result that strains bearing different morphological and physiological characters might be isolated from plants of the same species. Again, it might suggest that the adaptation of strains and their apparent ineffectiveness at times might be influenced by the degree of self- or cross-pollination. Further, it might bear a practical suggestion because one portion of the seed might symbiose adequately with one strain, while another portion might symbiose with still another strain, making it advisable for best results to employ two or more strains in a culture for inoculating the crop.

It should be suspected, from this viewpoint, that a species limited in its promiscuity is largely self-pollinating while a species that is promiscuous is highly cross-pollinating. Some data are available along this line, but more are needed. Certain species were grown in the greenhouse and others were studied under natural conditions for the purpose of observing this phenomenon as related to seed production. Greenhouse conditions may not offer the best environment for making such tests, and the results may be somewhat misleading because it is conceivable, among many possibilities, that the pollen may not germinate unless a high concentration of sugar is present. Such a concentration may not exist during short and cloudy days in the greenhouse. The setting of seed in the greenhouse, however, by certain species, which was accomplished without the aid of insects, was studied. *Cassia tora, C. oracle,* and *C. occidentalis,* species on which nodules have never been found, produced pods filled with seeds. *Trifolium fragiferum,* which was observed to symbiose with 7 of the 32 strains, produced seed from about 15 per cent of the flowers on each head. *Medicago lupulina,* which has been observed to symbiose with 13 of the 32 strains, produced some seed under these conditions. *Melilotus alba* was observed to nodulate with 11 of the 32 strains; *Lotus corniculatus,* which nodulated with 15 of the strains; *Medicago sativa,* which nodulated with 26 of the strains; and *Ononis pubescence,* whose symbiobility with the various strains was not determined, all failed to produce seed in the greenhouse. The last species should be like *Medicago sativa,* exhibiting a wide degree of promiscuity. Such observations, in addition to the data offered by Wilson present a close relationship between the degree of promiscuity and the degree of self- or cross-pollination.

If the promiscuity of a species or a variety is known, it should be an indication of the extent of cross-pollination. Likewise, if the extent of self- or cross-pollination is known, it should be an indication of the degree of promiscuity. In support of the ideas presented in these statements, the following tests were performed. Buckhout[1] described *Mimosa pudica* as a non-nodu-

lating species, but Fred, et al.[2] point out that this species sometimes bears nodules under greenhouse conditions. It should be suspected, therefore, that *M. pudica* is a highly self-pollinating plant and, as a result, should symbiose, perhaps, with a certain few strains of the rhizobia. Thus, its relations to the various strains were determined. The results showed that it symbiosed with the strains isolated from the following plants: *Apios tuberosa, Baptisia australis, Crotalaria spectabilis, Dalea alopecuroides, Robinia pseudo-acacia, Sesbania macrocarpa,* and *Swainsonia coronillaefolia,* or 7 of the 32 strains. This is 2 less than the number of strains with which *Glycine max* symbiosed, and in *G. max* Woodward found only 0.16 per cent natural crossing. On this basis, therefore, *M. pudica* should be a highly self-pollinating species. When this plant was grown to maturity in the greenhouse away from insects that might effect pollination, it appeared to produce well-filled pods with little or no difficulty.

Among the species studied in their natural surroundings is one which seems worthy of some remarks. It is *Cladrastis lutea.* Roots of numerous seedlings from 1 to 3 years old and those of mature trees were examined for nodules. About 100 seedlings grown on a variety of soils also were included. In no case was a plant found bearing a nodule. Bags of cheesecloth were, therefore, placed over the racemes of flowers to exclude insects. Excellent production of seed pods occurred, indicating that *C. lutea* is a non-nodulating and a self-pollinating species.

These data might be misleading, however, for *Cicer arietinum* was grown on Dunkirk soil at Cornell University for six consecutive years. Each year the roots were examined for nodules. During the last two years the seed and the soil in which the seed was planted were inoculated with a pure culture known to symbiose with Cicer under experimental conditions in the greenhouse. Nodules have never been found on the plants growing in the field. It is possible, therefore, that *Cladrastis lutea* was not observed and tested for nodulation under satisfactory conditions.

Relation of self- or cross-pollination of plants to promiscuity and to flagellation of strains of the rhizobia. A study was made of the morphology of those strains symbiosing with plants that exhibited slight if any promiscuity, such as *Cicer arietinum,* and the findings were compared with similar studies of strains from plants exhibiting pronounced promiscuity, such as *Phaseolus coccineus* or *Amorpha fruticosa.* Those strains from plants highly or entirely self-pollinating appeared to be largely monoflagellated, whereas those strains from plants exhibiting pronounced promiscuity appeared, in many instances, to be largely multiflagellated. All degrees of flagellation, therefore, appeared between the extremes, the degree conforming to some extent to the degree of self- or cross-pollination of the species. No perfect correlation appeared to exist, however, between the degree of promiscuity and the

degree of flagellation, for strains highly monoflagellated were found symbiosing also with species exhibiting a pronounced degree of promiscuity, whereas strains that were multiflagellated were seldom, if ever, encountered symbiosing with self-pollinating plants. This is a type of nonreciprocal relation the destiny of which appears to be closely associated with the degree of cross-pollination of the species.

These morphological studies of the strains from self-pollinating and cross-pollinating plants have laid the groundwork for a new approach to the study of adaptation of strains from one species to another. Some preliminary studies have been made. The strain direct from *Glycine max*, a highly self-pollinating plant, will occasionally symbiose with *Vigna sinensis*, a plant the cross-pollination of which is of about the same order as that of *G. max*. The opposite is true also; the strain from *V. sinensis* will occasionally symbiose with G. max. In both cases the strains are largely monoflagellated and if repeatedly passed through the plant for adaptation studies by nodulation and renodulation, few if any differences are detectable in appearance of organisms or in numbers of nodules produced after the several passages when compared with the results obtained by employing the original strains from each plant. Each strain becomes adapted to another species and appears to meet all the requirements of the plant. When such a strain has become adapted to another species and is then isolated from this species after adaptation, it might be designated as from this plant, even though its origin was from another species. Now that the strain from *Glycine max* has become adapted to *Vigna sinensis* it should bear a relation to other species similar to that of the strain originally isolated from *V. sinensis*. How far such adaptations can be extended when various strains are considered, is uncertain because some multiflagellated strains appear to symbiose almost exclusively with cross-pollinating plants. This may mean that adaptation can be more easily effected if the test in adaptation is undertaken with a full knowledge of the degree of cross-pollination of the plant and the degree of flagellation of the strain being employed. Results of a test of a similar nature are briefly described.

Many strains isolated from *Medicago sativa* will symbiose with *Melilotus alba*. A certain strain from *Amorpha fruticosa* symbiosed to a slight degree with *M. sativa* but did not symbiose with *M. alba*. From cross-pollination studies by Waldron[7] and by Kirk,[4] it appears that *M. sativa* is more cross-pollinating than is *M. alba*. It also appears more promiscuous than *M. alba*. *A. fruticosa* appears more promiscuous than either of the aforementioned species, but *M. sativa* is nearer *A. fruticosa* in this regard than is *M. alba*. After the strain from *A. fruticosa* had become adapted, at least in part, to *M. sativa* it then symbiosed with *M. alba* to some extent. Since the process of adaptation of a strain from one species to another occurs within the nodule, the strain from *A. fruticosa* might have been better adapted to *M. alba* than

it was, if it had been in the nodule of *M. sativa* for a longer time. It is conceivable that the environmental conditions will influence the speed and perhaps the permanency of the adaptation. The strains from *A. fruticosa* and *M. sativa* which were employed in this work appear morphologically somewhat similar.

SIGNIFICANCE OF THE FINDINGS

The evidence bearing on symbiotic promiscuity between the legumes and their associated organism is more tangible than that bearing on the explanation for its cause. The widespread promiscuous relations of many plants with the various strains of Rhizobia suggest that the present methods of grouping plants in relation to their association with certain strains does not account for the facts. A study of the strains from *Amorpha fruticosa* substantiates this suggestion, for strains were isolated from *A. fruticosa* that appeared better adapted to other plants than to Amorpha. Such promiscuity makes it difficult if not impossible to draw definite boundaries around a plant and say that it belongs in a certain group. The facts available indicate, for many plants, at least, that there are one or more strains that meet every requirement of the plant, strains that are a little less well adapted to the plant, and still other strains less and less well adapted, until some are found which do not symbiose with the particular plant at all. As the strains encountered appear less and less well adapted to the particular plant, the results indicate that they appear more and more adapted to some other plant. This bridging over from one species, or variety, to another is the region of promiscuous relations, a region of reciprocal and nonreciprocal symbiotude. Apparently no strain is specific. In view of the existence of such promiscuous relations, specificity can be only relative. This applies also to the proposed plant-bacteria groups: the lack of specificity makes them so relative that they cannot be used. Who can say, therefore, that this or that strain is typical for this or that plant? A strain may appear perfectly adapted to plants that are representatives of two or more plant-bacteria groups. A plant like *Cicer arietinum* may appear to symbiose with only one strain, yet the strain may be found associating with other species. If the strain should be found associating with another species and tested for its symbiobility with Cicer, then Cicer would not be placed in a group by itself. This should not be taken to mean that a strain which is well adapted to a certain plant possesses no promiscuous characters, for no strain has been encountered that will symbiose with just one plant. The strain from Cicer has been considered by researchers to be specific. Upon tests it was found to symbiose with 3 species out of 30. If previous workers had included in their tests for specificity enough species, Cicer would not have been placed in a plant-bacteria group by itself. This applies also to each strain. From the many strains that exist in nature it

appears possible that one or more may be specific for a certain plant, but so far no such strain has been encountered.

The evidence submitted relating to the degree of cross-pollination and promiscuity offers a new approach to the study of legume bacteria, non-nodulation and nodulation of plants. With this view it has been possible to predict rather accurately, in the dozen or more cases that have been studied, the degree of promiscuity possessed by certain plants. Restricted promiscuity has always been encountered in plants that are highly self-pollinating; and almost unhampered promiscuity, in plants that are highly cross-pollinating. With this viewpoint it has been posssible to suggest an explanation for many conflicting statements in the literature, for reciprocal and nonreciprocal relations between the plant and the various strains of the rhizobia, for the nonoccurrence of nodules on certain species, and for the variations encountered in studies of the morphology and of the physiology of the strains. This is particularly true as regards the findings of various researchers relating to flagellation. Certain investigators have reported the strain from a certain plant to be monoflagellated, whereas other investigators have reported the strain from the same species to be multiflagellated. The recognition of promiscuity offers a satisfactory explanation for the findings of both groups.

ABSTRACT

In this study of symbiotic promiscuity in the Leguminosae plantlets from sterilized seed were grown in flasks on a sterilized substrate that was wetted with a solution containing in suspension the desired strain of Rhizobium. This made it possible to grow the plantlets and the strain together to the exclusion of other strains and to observe whether nodules occurred on the roots. These were the criteria of symbiosis. In addition to reporting tests with strains from each plant-bacteria group, 46 strains were isolated from one species and employed in tests to show that promiscuity is widespread. These strains exhibited adaptation not only to *Amorpha fruticosa*, from which they were isolated, but also to many other species which are representatives of several plant-bacteria groups. Also growth of the strains from *A. fruticosa* on media revealed that the strains, as measured by rate of growth, were as variable as that which may be expected from strains from plants representing each of the 16 or 20 plant-bacteria groups that were projected. Data were presented, also, to show that this promiscuity is closely associated with the degree of self- or cross-pollination and that by knowing the degree of cross-pollination it is possible to predict, to some degree, the extent of promiscuity inherent in a plant. It was observed in connection with the flagellation of strains that plants which are entirely self-pollinated may be also non-nodulating, that plants which are restricted in cross-pollination symbiose mostly with strains highly monoflagellated and that plants which are obliga-

torily cross-pollinated usually symbiose best with strains highly multiflagellated although such plants may symbiose with strains possessing variable degrees of flagellation.

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BACTERIA ASSOCIATED WITH *GLEDITSIA TRIACANTHOS* L.

LEWIS T. LEONARD

Soil Microbiology Investigations, Bureau of Plant Industry, U. S. Department of Agriculture, Washington, D. C., U. S. A.

THERE are species among the legumes which do not under any known circumstances produce typical leguminous nodules on their roots. These species are listed under the genera Cassia, Gymnocladus, Cercis, Gleditsia, and Ceratonia in the subfamily, Caesalpiniaceae. Lack of nodules is not necessarily proof that these species do not possess the ability to fix atmospheric nitrogen in conjunction with loosely or otherwise associated bacteria or even other types of organisms, but it does leave room for considerable doubt. The species to which the work reported in this paper is confined is *Gleditsia triacanthos* L., honey locust, a tree indigenous to the United States, its range extending from New York to Texas. In addition to its widely scattered natural distribution it has been extensively propagated and planted as an ornamental. Rather recently an attempt has been made to promote it as a forage tree on the basis of its plentiful supply of pods, which are acceptable to stock as food. This endeavor has raised the question whether the plant harbors nitrogen fixing bacteria within its roots.

Some of the earlier investigators, including Nobbe, Schmid, Hiltner and Hotter,[11] Morck,[10] Harrison and Barlow,[5] and Leonard,[7] observed that Gleditsia roots did not develop nodules. Buchanan[1] apparently thought this legume produced nodules. McDougall,[9] after a study of the root hairs of Gleditsia, confirmed the opinion of Nobbe et al.[11] that the lack of nodules was due to the thick walls of the root hairs. Feher and Bokor[2] mention bacteria in reference to this legume but give little evidence to substantiate any claim to nitrogen fixing qualities. Friesner,[4] on the other hand, in a preliminary report which unfortunately has remained in preliminary status, observed that on seedling trees thickened cylindrical swellings occur at the ends of the smaller rootlets. From superficially disinfected swellings of this sort she obtained upon plating "typical whitish glistening colonies of a stringy, sticky consistency" from very low dilutions of crushed material.

Pot tests including pure culture and soil suspension inocula are reported to have confirmed the presence of the same organisms in the soil and in the

root tissues. Unfortunately, the only possible reference to evidence of nitrogen fixation is extra root development in the inoculated plantings. The organism isolated is described as to microscopic and macroscopic appearance and reaction to various culture media. To throw more light on this question, attempts to isolate organisms from cylindrical swellings have been made, and vegetative tests have been used to determine whether appreciable nitrogen fixation takes place.

ORIGIN OF SAMPLES

Through the Soil Conservation Service* many samples of Gleditsia of different sizes and ages were obtained from various parts of the country. Samples from a distance were packed in moist moss, covered with waterproof paper, and shipped by mail. Nearby samples were collected personally. Table 1 shows information concerning the samples used in this investigation.

TABLE 1

ORIGIN AND CONDITION OF SEEDLING SAMPLES OF GLEDITSIA

Sample Number	Origin	Condition	Species
2981	Beltsville, Md.	In leaf	triacanthos inermis
2982	Beltsville, Md.	In leaf	triacanthos inermis
2983	Perulack, Pa.	In leaf	triacanthos
2997	Albuquerque, N. M.	Dormant	triacanthos inermis
2998	Albuquerque, N. M.	Dormant	triacanthos inermis
2999	Mandan, N. D.	Dormant	triacanthos inermis
3000	Pullman, Wash.	Dormant	triacanthos inermis
3001	Beltsville, Md.	Dormant	triacanthos
3004	Manhattan, Kans.	Dormant	triacanthos inermis
3005	Manhattan, Kans.	Dormant	triacanthos
3008	Stillwater, Okla.	Dormant	triacanthos
3009	Stillwater, Okla.	Dormant	triacanthos inermis
3014	Shiprock, N. M.	Dormant	triacanthos
3015	Shiprock, N. M.	Dormant	triacanthos
3016	Sibley, La.	Dormant	triacanthos

SELECTION OF MATERIAL

When received, the samples were thoroughly washed and examined for the presence of terminal swellings analogous to those described by Friesner.[4] On very few samples was anything of that nature found, and there was no indication of consistent occurrence as is the case with typical leguminous plant nodules. Pieces of rootlets, however, were selected for plating. It is quite possible the rootlets in some instances were torn off in the digging, but

* Courtesy of Harry A. Gunning.

most of the plants received had at least part of their small rootlets. It is also a possibility, at least with reference to the dormant samples, that the swellings may have disappeared in the manner of leguminous nodules. Since the growing specimens failed to exhibit more characteristic swellings than the dormant ones, it is highly probable that dormancy was not an influence.

The material selected for plating was washed through four 250-cc. lots of sterile tap water. In some instances platings were made from material which had been washed in this manner. In others, after being washed, the material was placed in a liberal volume of mercuric chloride solution and, with shaking, exposed for varying lengths of time. Immediately after the removal of excess disinfectant, a 250-cc. portion of sterile tap water was added and thoroughly shaken. It was then removed and another portion added. This process was continued two more times.

Each individual portion of a rootlet was put into a test tube containing about 10 cc. sterile tap water, crushed, and shaken to mix. Inoculating material for plates consisted of drops and 1-cc. portions of the original dilution and similar amounts from a 1:100 dilution of the original concentration.

Plating was done with several media at first and finally with Lipman's agar[4] and a medium with the basal materials of yeast water-mannitol agar, except that only 20 cc. yeast water per liter was used, and in addition 20 cc. each of asparagus extract, potato extract, sauerkraut juice, and 1 per cent calcium gluconate per liter.

Although similar colonies did not appear consistently, as is common in the plating of nodule bacteria, selections were made of those having a resemblance to the described colony, and these were transferred to tubes. They were purified by replating once. A summary of the data concerning plating is given in table 2.

Of 73 samples plated, 51 were disinfected. From the disinfected material 10 cultures were selected, and from the 22 washed samples, 26 cultures were selected. Out of 22 samples disinfected with 1:500 $HgCl_2$ and 8 samples treated with 1:1000 $HgCl_2$, no cultures were obtained. Five cultures each were obtained from material disinfected with 1:500 and 1:1000 $HgCl_2$.

Since the succulent nodules of legumes will tolerate a much longer exposure to disinfectant of the strengths used and yet give satisfactory isolations, it is not probable the bacteria in the interior of the Gleditsia rootlets were injured by the treatment given. If bacteria were in the root tissues, it is evident that they were very scarce, since even in the greatest concentration of disinfected material, only a few colonies of bacteria developed. A much greater number of colonies was found on plates from dilutions of the undisinfected material. From the treatments given and the cultures obtained, it seems apparent that degree of surface disinfection is a deciding factor in the number of colonies obtained.

TABLE 2

ISOLATIONS FROM ROOT SWELLINGS OF GLEDITSIA

Sample number	Number of roots plated	Number of media used	$HgCl_2$ concentration	Time of exposure	Roots disinfected	Cultures obtained	Roots washed	Cultures obtained
				minutes				
2981	6	5	1:500	5	4	0	2	4
2982	6	5	1:500	5	4	0	2	4
2983	8	5	1:500	5	5	0	3	4
2997	6	2	1:500	5	3	1	3	6
2998	6	2	1:500	5	3	2	3	2
2998	3	2	1:1000	2	3	2	0	0
2999	6	2	1:500	5	3	0	3	2
3000	6	2	1:500	5	3	0	3	0
3001	6	2	1:500	5	3	0	3	4
3004	3	2	1:1000	2	3	1	0	0
3005	3	2	1:1000	2	3	2	0	0
3008	3	2	1:1000	2	3	0	0	0
3009	3	2	1:1000	2	3	0	0	0
3014	3	1	1:1000	2	3	1	0	0
3015	3	1	1:1000	2	3	1	0	0
3016	2	1	1:1000	5	2	0	0	0
Totals	73				51	10	22	26

IDENTIFICATION AND ELIMINATION OF CULTURES

Certain selected colonies were suspected of being *Achromobacter radiobacter*. These were placed on the soil-extract glycerine nitrate of Smith[12] and made typical growth. They were then put into potato and milk as suggested by Löhnis and Hansen,[8] and their identity was further confirmed. Six cultures in all were found to be *Achromobacter radiobacter*, three from disinfected and three from undisinfected rootlets. As a result of this elimination 7 cultures from disinfected material and 23 from the washed rootlets remained. Two of the Radiobacter cultures originated from material treated with 1:500 $HgCl_2$ for 5 minutes and one from a treatment involving 1:1000 $HgCl_2$ for 2 minutes. It is evident, in some cases at least, that Radiobacter can survive the ordinary means of disinfection with mercuric chloride. Further consideration of the identification of cultures from undisinfected roots was stopped at this point.

Of the remaining seven organisms from disinfected roots, only three made a viscid, stringy growth on Lipman's agar, and these made a filiform smooth streak on both nutrient and Lipman's agar. Growth in no case was ridged. None of the cultures liquefied gelatin. Except for the viscid growth in three cases, these growth characteristics are not in accord with Friesner's description,[4] and therefore the results are not confirmatory of her work.

NITROGEN FIXATION TRIALS

Statements attributing nitrogen fixing abilities to Gleditsia and other non-nodule forming members of the Caesalpiniaceae have been made by popular writers. There seems to be no work available which confirms these opinions. They are probably based on the assumption that nitrogen fixation by bacterial association is a fixed function of all legumes. In order to determine whether nitrogen fixation does or does not take place, an experiment was prepared involving seed of *Gleditsia triacanthos inermis*.

Seeds were prepared for germination by placing one part in three parts boiling water and allowing this to cool. Then, 1:500 mercuric chloride solution was added and shaken with the seed for 5 minutes. Following disinfection the seeds were washed four times with 250-cc. portions of sterile tap water. Finally they were placed on the surface of 0.6 per cent agar in petri dishes and incubated at 20° C. for several days. From these lots of seedlings selections were made for planting the experiment.

Tubulated jars as described by Kellerman[6] were filled with river sand to within a half inch of the bottom of the tubulature. This sand was moistened with modified Crone's solution,[3] the tubulature was closed with cotton, and the open top of the jar was covered with cellophane anchored by adhesive tape to the glass. The jars and their contents were sterilized for 2 hours at 17 pounds steam pressure.

INOCULA

The pure culture inoculum was prepared by mixing a loopful of each of 12 cultures from the first three undisinfected samples with 300 cc. sterile tap water. The combination was thoroughly shaken. Half of the mixture was placed in the autoclave and sterilized 5 minutes at 105° C.

For soil inoculum 10-gm. portions of soil from around the roots of each of three mature trees growing in nearby Virginia, were placed in 300 cc. sterile water and mixed. Half of this suspension was also sterilized.

For the crushed root inoculum small rootlets from young trees at Beltsville, Maryland, and from an old tree at Rosslyn, Virginia, 6 gm. in all, were crushed in a mortar and added to 300 cc. sterile water and shaken thoroughly. Half of this mixture was autoclaved also.

Thus three types of inocula with their respective controls were obtained. Before use, these were put into sterile petri dishes, and seedlings were immersed in them before planting. Seedlings were placed in a hole in the sterile sand and tamped so as to cover the root and hold the plant in place. In addition to control jars containing plants treated with sterilized inocula, some jars were prepared with untreated seedlings.

All operations were performed with sterilized implements, and every prac-

tical effort was made to prevent contamination of the jars during the planting and watering periods, the only times of exposure. After being placed in the greenhouse, the jars were given 15 hours of light each day, which included the normal daylight and the electric light applied in the evenings and during cloudy weather. In the approximate 100-day duration of the test, water was added three times to each jar.

OBSERVATIONS ON GROWING PLANTS

There were no variations in the color of growing plants which could be definitely attributed to treatments. In general, the leaves were dark green, and in some instances the lower leaflets turned yellow. On the basis of size, with consideration of original seedling variability, there was nothing superficial to differentiate the treated plants from the control plants.

At the end of the experiment the plants were gently eased from their respective jars so as not to break off any parts of the roots. Root length and size varied considerably between plants from the same treatment, and since the roots of the untreated plants attained a greater average length than did those of the treated plants, no confirmation of Friesner's[4] observations on roots is available. No evidence of cylindrical swellings at the end of rootlets was noticed.

The plants were washed as free from sand as possible, dried, and weighed. The nitrogen analyses* were made on the whole samples; by this means possible errors due to sand clinging to the roots were avoided. Data concerning dry weight and nitrogen content are shown in table 3.

TABLE 3

DRY WEIGHTS AND NITROGEN CONTENTS OF GLEDITSIA SEEDLINGS

Treatment	Number of plants	Total dry weight	Average dry weight	Total nitrogen	Average total nitrogen
		gm.	*gm.*	*mgm.*	*mgm.*
Control 1	10	3.095	0.31	64.0	6.40
Pure culture	10	3.105	0.31	64.0	6.40
Soil suspension	6	1.415	0.24	29.6	4.93
Root suspension	8	1.905	0.24	45.8	5.72
Pure culture (sterile)	10	3.015	0.30	61.2	6.12
Soil suspension (sterile)	9	2.470	0.27	51.4	5.71
Root suspension (sterile)	9	2.410	0.27	50.8	5.64
Control 2	4	1.115	0.28	28.0	7.00

Considered individually or collectively, the control plants are better than the treated plants from the standpoint of dry matter and nitrogen content;

* Analytical work done by Daniel Ready.

and therefore only one conclusion can be drawn, that the inocula were without positive effect.

CONCLUSIONS

Under the circumstances of this experiment, the following conclusions seem reasonable:

There is no consistent formation of terminal cylindrical root swellings on seedlings of *Gleditsia triacanthos*.

Bacteria in the interior of the roots, if any, are very rare.

No isolates corresponded to the organism previously described.

Apparently isolations were surface organisms which by some means were untouched by the disinfectant.

Treatments involving pure culture, soil, and root inocula gave no evidence of benefit when judged by color, dry weight, root development, or nitrogen content.

No basis is evident for the statement that Gleditsia is a nitrogen-fixing plant.

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SOME SOIL FACTORS IN NITROGEN FIXATION BY LEGUMES*

WM. A. ALBRECHT

University of Missouri, Columbia, Missouri, U. S. A.

BY MEANS of a plant medium of colloidal clay[3] on which various nutrient cations and anions are adsorbed, it is now possible to put the soil phase of nitrogen fixation by legumes under chemical control as accurate as that possible by the water culture methods for plants, or the agar cultures for isolated bacteria. Rather than establish our ideas about nitrogen fixation by piecing together the information gained by each of these performers in isolation, we can now interpret nitrogen fixation by observing the soil, the plant, and the bacteria, all performing cooperatively under accurate chemical control. Though colloidal clay mixed into quartz sand is not truly a soil, yet its simulation of such is so close as to help us evaluate more exactly the significance, in the process of nitrogen fixation, of (a) the soil texture, (b) the pH, or degree of acidity, (c) the degree of calcium saturation of the clay, (d) the fertility in terms of organic matter content, (e) the phosphorus mobility, (f) the exchangeable magnesium content, and (g) the exchangeable potassium content of the soil.

SOILS OF "HEAVY" TEXTURE GIVE INCREASED NITROGEN FIXATION

It has been observed often that legumes may be growing successfully on a heavy clay soil the degree of acidity of which is such as would cause their failure in most soils. Experiments with colloidal clay media show that increasing amounts of clay at certain degrees of calcium saturation give improved nitrogen fixation and better growth by the legume.[1] When electrodialyzed colloidal clay that was originally at pH 3.6 and was titrated by calcium hydroxide so as to give a range in pH from 4.0 to 6.5 by intervals of 0.5, was added in increasing amounts to a constant quantity of sand, the growth of soybeans showed improvement. Such growth improvement corresponding with more clay, or with a heavier soil texture, occurred for the clays of different acidities when these were pH figures above 4.0. At this

* Contribution from the department of soils, Missouri Agricultural Experiment Station Journal Series No. 611.

latter degree of acidity, very little growth occurred. These results point to the clay fraction as a significant soil factor in the process of nitrogen fixation.

The efficiency with which the soybean plants, for example, can withdraw the calcium from the exchangeable supply in the calcium-saturated colloidal clay was determined. Plants given increasing amounts of such clay in sand were analyzed,[4] and their nitrogen fixation was determined as related to the efficiency with which they consumed the exchangeable calcium from the clay when they were restricted to other nutrients supplied only in the seed. The results given in figure 1 point out that even with an ample but

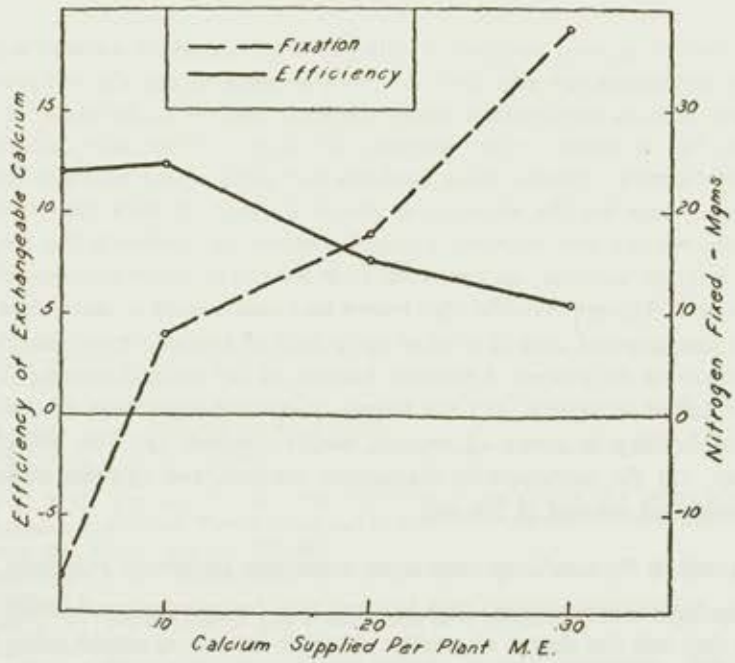

FIG. 1. Nitrogen fixation as related to efficient use of exchangeable calcium with increasing clay content of soil.

constant total supply, the maximum efficiency of such calcium use was about 12.5 per cent. This efficiency was lower as the fixation was higher, and as a result, with the most effective nitrogen fixation only about 6.5 per cent of the exchangeable calcium supply was taken, or served the plant.

For these legumes the exchangeable calcium would not be available *in toto* but only to about one-sixteenth during these 5 to 6 weeks of growth. This suggests that before we can use chemical measures of the exchangeable nutrient supplies in the soil as indexes of fertility, it is necessary to determine the efficiency with which these nutrients in exchangeable form serve the different crops.

THE DEGREE OF ACIDITY, OR pH OF THE SOIL, AND NITROGEN FIXATION

The foregoing studies served to emphasize the significance of the degree of soil acidity and the nature of the colloidal clay complex as factors in legume growth and nitrogen fixation. In the first case cited, nitrogen fixation during a 6-week period of growth failed at all pH figures below 5.5, even with increasing amounts of clay in which the total absorbed, or exchangeable, calcium was no greater than 0.2 m.e., or 4 mgm., per plant. Nitrogen fixation occurred, however, and was improved with increasing amounts of clay—and therefore of calcium—at pH figures of 5.5 and higher. With the increasing nitrogen fixation there were larger amounts of calcium in the crop as taken from the clay. These were larger both as the amount of clay increased and as the pH figure increased.

There is an inclination to give emphasis to the relation between the pH figure and the process of nitrogen fixation. The plant growth and this fixation process were influenced more, however, by the amount of clay—and therefore of the calcium offered—than by the pH. In fact, doubling the amount of clay improved the process as much as, or more than, a reduction of 1.0 pH, or by ten times in the hydrogen-ion concentration. Thus, since the nature of the clay is such that an increased degree of acidity, or a lower pH figure, represents less calcium, then, in reality, nitrogen fixation is related to acidity, or pH, only as this represents a decreasing supply of calcium as a plant nutrient. This statement holds for the studies with soybean plants, the isoelectric point of which was at approximately pH 5.5. At degrees of acidity less severe than this, the soil calcium moved into the plant for improved growth and nitrogen fixation. At degrees of acidity more severe than pH 5.5, the plants took no calcium from the soil and fixed no nitrogen. On the contrary, they lost bases, or positive ions, from the seed supply to the clay, and a growth resulted with plants of less calcium content than that originally represented by the seed. Thus, it is true that the pH can be so low that no nitrogen fixation occurs, but the calcium deficiency is the true cause and the pH merely a contemporaneously associated variable. This process cannot be expected unless significant increase in the calcium content of the plant is brought about by the delivery of this element to the plant by the soil.

DEGREE OF CALCIUM SATURATION OF THE CLAY AND NITROGEN FIXATION

The nature of the colloidal clay as a complex which can absorb cations—possibly anions—and exchange them with other ions, suggests that the influence of increasing H-ion concentration on nitrogen fixation by legumes may be an effect of the reciprocal, namely, the decreasing degree of calcium saturation, on this Beidellite mineral. A test was made on the influence of the

degree of calcium saturation on nitrogen fixation by soybeans. Saturation degrees of 40, 60, 75, 87.5, and 97 per cent of the capacity of the clay for calcium were used when the accompanying ions were hydrogen, giving clays of varying acidity, or when they were magnesium, potassium, or barium, all giving purely neutral clays. Amounts of the clays were taken to give constant amounts of exchangeable calcium per plant.

All of the visible plant characters demonstrated the importance of the degree of calcium saturation, but there was no significant influence of the accompanying ions when these were the non-nutrients, namely, hydrogen and

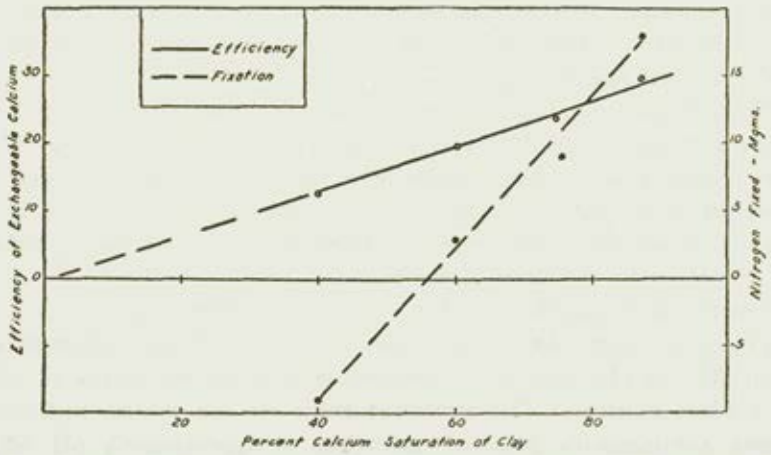

FIG. 2. Nitrogen fixation as related to the efficiency of calcium use at different degrees of calcium saturation of the clay. (H, Ba, Mg, K as reciprocal ions. Mean results of six trials.)

barium. Here again the effectiveness with which the calcium was delivered served as a measure of the plant performance as a nitrogen fixer. Increased degree of saturation gave greater nitrogen fixation, irrespective of the accompanying cation. Along with it went a greater percentage use of the total calcium offered, or a larger supply of exchangeable calcium was delivered to the plant, as shown in figure 2.

A higher efficiency in the use of the calcium was shown in these trials than in the preceding cases. This efficiency was related to the degree of saturation as illustrated by a straight line in figure 2. This line demonstrated a mean ratio of 0.3215 for the degree of calcium saturation to the percentage of utilization, or a tangent of an angle of 17.8°, for the increased efficiency by calcium with increasing degree of clay saturation by it. It shows forcibly that calcium from the same original total supply is delivered into the plants to a much larger extent when it is on a nearly saturated clay than when on one only partly saturated. As an illustration, the 200 mgm. of calcium distributed on enough clay to saturate it to but 40 per cent, delivered only

21.4 mgm. to the plants. When this same total amount was put on less clay so as to saturate it to 97 per cent, the delivery to the plant amounted to 58.2 mgm., or 2.7 times as much.

As the degree of calcium saturation of the clay becomes lower, more total exchangeable calcium must be present in the soil to be equally as effective as that at the higher degrees of saturation. More exchangeable ions can be provided by supplying more clay. As an illustration, let us suppose that the situation in a silt loam with 15 per cent clay is the same as that for 87.5 per cent calcium saturation in these trials where 29.3 per cent of the total exchangeable calcium moved into the plant. Then let us calculate how much clay equivalent would be required to deliver the same amount of effective calcium, and what quantities of exchangeable calcium would be represented in each of the different degrees of calcium saturation and percentage efficiency

TABLE 1

PERCENTAGES OF CLAY REQUIRED IN A SOIL AT DIFFERENT DEGREES OF SATURATION TO DELIVER AS CONSTANT AN AMOUNT OF EFFECTIVE CALCIUM AS WAS FOUND FOR 87.5 PER CENT SATURATION

Degree of saturation	Exchangeable calcium		Effective calcium	Clay required	Clay in soils
	Per cent used	Pounds per acre	Pounds per acre	Pounds per acre	
Per cent					*Per cent*
87.5	29.3	3,412	999.8	300.000	15.00
75	23.8	4,200	999.8	430.769	21.53
60	19.7	5,075	999.8	650.641	32.53
40	12.3	8,128	999.8	1,563.076	78.15

cited. Such data are given in Table 1 for the Beidellite clay of which the total exchange capacity was taken as 65 m.e. per 100 gm. of clay.

Since the effectiveness of exchangeable calcium decreases decidedly as the percentage saturation decreases, the amount of clay demanded to provide constant amounts of effective calcium, according to these studies, rises so rapidly that a silt loam at 87.5 per cent calcium saturation is as effective in providing calcium as is a clay soil with 78 per cent clay at 40 per cent saturation. These figures for efficiency of calcium at different degrees may be far from the actual truth in the field, but they serve, nevertheless, to emphasize the fact that calcium delivery to the plant is much more efficient at the higher degrees of calcium saturation of the clay.

These results showed that growth of the plants occurred at all these degrees of saturation by calcium, but nitrogen fixation became significant only at the

higher degrees. Thus, plants may be growing but failing to fix nitrogen because of a deficient degree of saturation rather than because of a deficiency in the total supply of this element in exchangeable form. The results suggest that nutrient additions to the soil might well be applied so as to saturate a limited soil volume, with consequently more efficient use by the plants than when applied through a greater soil volume with correspondingly lower degree of soil saturation. This suggests drilling rather than mixing fertilizers with the soil.

ORGANIC MATTER CONTENT OF THE SOIL AND NITROGEN FIXATION BY LEGUMES

Since it is now recognized that the mineral clay complex of the soil unites chemically with residues from organic matter decomposition,[5] an attempt was

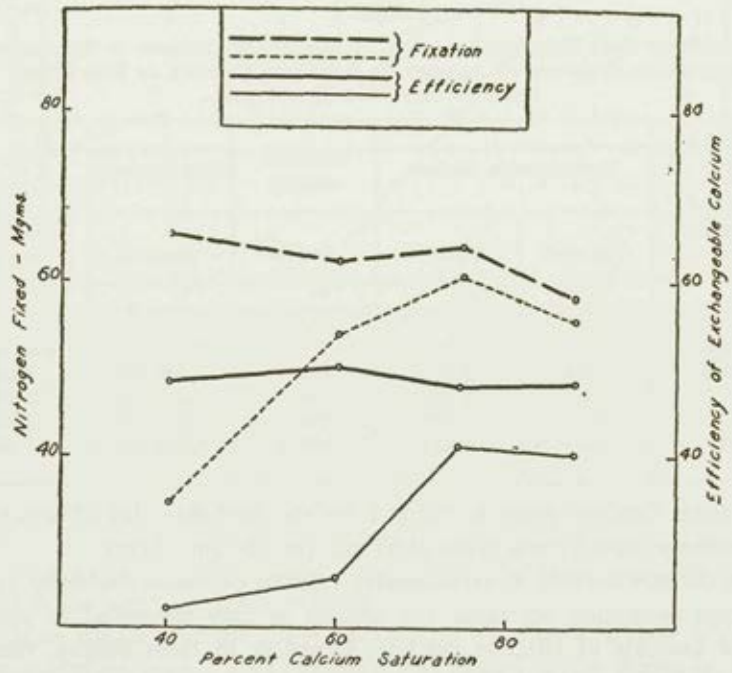

FIG. 3. Nitrogen fixation as related to the degree of calcium saturation when reciprocally accompanied by methylene blue or hydrogen. (Heavy line—methylene blue; light line—hydrogen.)

made to simulate a clay having part of its saturation capacity taken by calcium and part by organic matter. This was brought about by giving the clay variable degrees of calcium saturation as previously cited, while the remainder of its capacity was given methylene blue, an organic compound

of great molecular size and weight. Soybeans were grown on this medium, delivering the same total exchangeable calcium as that in the preceding test, and their nitrogen fixation was noted. They were compared with a series using hydrogen as the ion accompanying the calcium, with the results given graphically in Figure 3.

The most noticeable result was the fact that the efficiency of calcium use by the legume was not related to the degree of saturation. Rather, the efficiency was almost a constant (48.5-51.0 per cent, mean 49.4 per cent) for all degrees of saturation. Here with the larger organic cation accompanying the calcium, the efficiency of calcium use was more closely related to the total supply of exchangeable calcium than was the case when less strongly adsorbed ions, H, Ba, Mg, or K, accompanied the calcium. Such results suggest that, probably, when a soil is liberally supplied with partly decomposed organic matter, this may exercise beneficial effects on nitrogen fixation indirectly. These may be exercised through the replacement of the hydrogen on the clay by a very large organic molecule, the high adsorption energy of which holds it so firmly that only calcium and other more mobile cations move from the clay into the plants. Such a view may help in the understanding of why certain degrees of acidity in soils of decidedly higher organic matter content are less disturbing to nitrogen fixation by legumes than are these same degrees of acidity in soils almost devoid of organic matter.

PHOSPHORUS MOBILIZATION AND NITROGEN FIXATION

The phosphate cation may be adsorbed by the colloidal clay, hence the clay medium lends itself to the study of this anion, as well as to cations, as a factor in nitrogen fixation by legumes. The adsorption of the phosphorus is influenced by the adsorbed calcium. Studies of the effect of phosphorus on nitrogen fixation by the colloidal clay medium for soybeans show that the mobility of the phosphorus at low levels and its activity in plant growth and nitrogen fixation are linked with the calcium behavior.

Variation in the phosphorus levels of the colloidal clay were without effect on the nitrogen fixation at low levels of calcium. Unless significant amounts of the latter element were present, the plants failed to metabolize into their tissues even the phosphorus present in the seed. Unless the entire phosphorus content of the seed moved into the plants, the latter failed to fix nitrogen during a growth period of 6 weeks. When ample calcium was applied, the quantity of phosphorus in the seed was enough to give significant nitrogen fixation during this period. Four trials were made with increasing amounts of calcium per plant to note the mobilization of the seed phosphorus and its possible relation to the nitrogen fixation. The mean results of the seed phosphorus metabolized in the plants as related to the calcium content of the crop and to the nitrogen fixation activities are shown graphically in Figure 4.

It is interesting to note that with a mean total of 49 mgm. of phosphorus in the seed at the outset, much of this was returned by the plants to the soil unless 0.2 or more m.e. of calcium per plant was present. Further, significant nitrogen fixation occurred only when the total seed phosphorus was moved into the plant and when none was returned to the soil. Thus, there was a close relation between the phosphorus supplied to the plant and nitro-

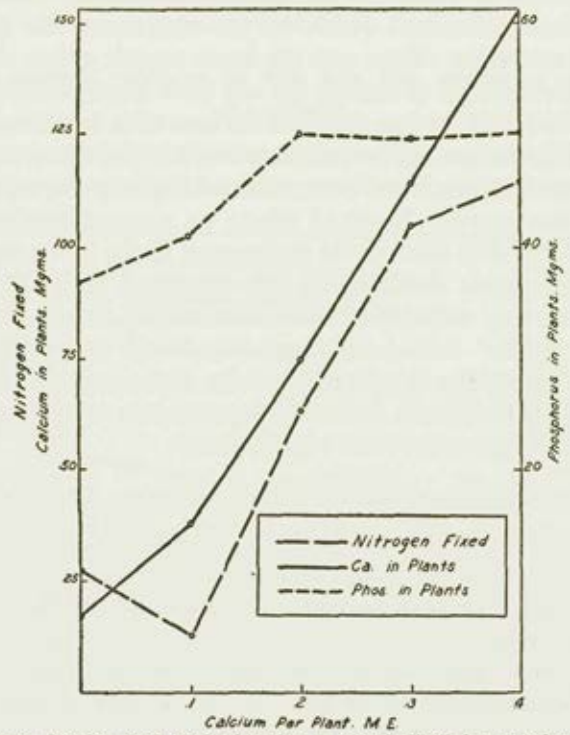

FIG. 4. Nitrogen fixation and seed phosphorus metabolized into soybean plants as related to calcium level provided.

gen fixation. These two, in turn, were related to the amount of calcium supplied.

Chemical studies of legumes in the field[2] point to the increased mobilization of phosphorus from the soil into the crop by the calcium treatments. This increase amounted to as much as trebling the phosphorus taken from the soil. It held true for corn, a nonlegume, as well as for lespedeza, a legume.

Thus, if increased phosphorus is required for improved nitrogen fixation, and this increased phosphorus in the plant is impossible without a significant supply of calcium, then the latter improves nitrogen fixation, in part at least, through its mobilization of phosphorus into the plant.

It seems, then, that the three elements nitrogen, phosphorus, and calcium are closely connected with nitrogen fixation by legumes. The first two might readily be related to nitrogen fixation, since both occur as constituents of proteins. That calcium should be associated with protein production not so much as a constituent of the final product but as an agency in its production is not a common belief. Recent relation of this element in the contraction bands of muscular tissue[6] and its persistent correlation with nitrogen fixation in these studies, suggest that this element may play a role in the production, renewal, or other metabolic phases of protein.

EXCHANGEABLE MAGNESIUM AND NITROGEN FIXATION

When a variable supply of magnesium was used in connection with a constant level of calcium on colloidal clay to determine the significance of mag-

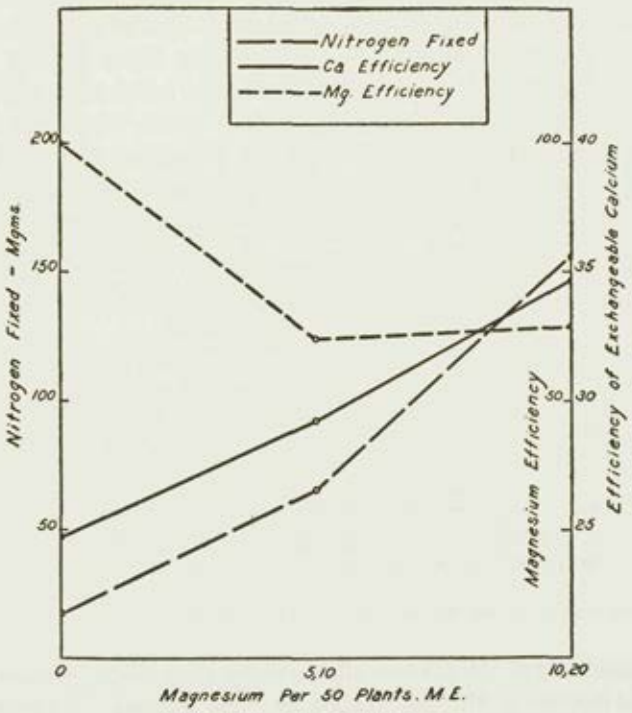

FIG. 5. Nitrogen fixation as influenced by magnesium effects on calcium efficiency.

nesium in nitrogen fixation, this element showed a decided influence on the growth and nitrogen fixation by soybeans, as illustrated graphically in Figure 5. That this influence is not directly related to the amounts of magnesium taken by the plants is indicated by the fact that the efficiency with which the

magnesium was used is not related to the nitrogen fixation. The magnesium increase, however, gave an increased efficiency in the absorption, by the plants, of calcium from the constant supply of this element at two different levels. This increased efficiency of calcium paralleled the increased nitrogen fixation. The increased total calcium consumption agreed with the increase in plant growth. Such relationships were not evident between the efficiency with which the magnesium was taken and the nitrogen fixation, or between this latter process and the total or percentage of magnesium in the crop. It would seem more logical, then, to believe that magnesium was instrumental in making the calcium effective in the process of nitrogen fixation, rather than that magnesium is directly active in this performance.

That this seems the proper conclusion is indicated by the fact that when magnesium supplemented the calcium, the efficiency of the latter was increased decidedly. The concentration of calcium in the crop was also lower as this element was more effective in nitrogen fixation. Use of atmospheric nitrogen occurred at a calcium concentration in the plant tissue of 0.45 per cent, a concentration lower than those in previous trials, which were commonly 0.50 to 0.55 per cent. At this lower concentration within the plants, in this case using magnesium, the phosphorus and the potassium from the seed were completely metabolized into the plant at the same total amount of calcium per plant required for this in previous trials giving less total growth. Thus,

TABLE 2

LOWERED NITROGEN CONCENTRATION IN SOYBEANS* AS RELATED TO APPARENT POTASSIUM SUBSTITUTION FOR CALCIUM

Exchangeable cations			Crop weight	Nitrogen		Magnesium		Calcium		Phosphorus		Potassium		$\frac{K}{Ca}$	Calcium efficiency
Mg	Ca	K													
m.e.	*m.e.*	*m.e.*	*gm.*	*per cent*	*mgm.*	*per cent*	*mgm.*	*per cent*	*mgm.*	*per cent*	*mgm.*	*per cent*	*mgm.*	*per cent*	*per cent*
5	10	0	14.207	2.86	407	.36	52	.74	105	.25	39	1.01	150	1.36	49.5
5	10	5	14.592	2.56	372	.36	54	.32	46	.18	26	1.90	285	5.93	21.2
5	10	10	17.807	2.19	390	.30	55	.27	48	.14	25	2.15	384	7.96	22.1

* Seed content in mgm.: N = 364, Mg = 16.7, Ca = 12.2, P = 39.4, K = 171.

the supplementing of the calcium allotment by magnesium increased the effectiveness of calcium in nitrogen fixation by these legumes. We may, therefore, consider the exchangeable supply of magnesium in the soil as a factor indirectly significant in nitrogen fixation.

VARIABLE POTASSIUM SUPPLIES AND NITROGEN FIXATION

When soybeans were grown on colloidal clay with such constant amounts of calcium and magnesium as were previously found effective, but which were

supplemented by variable amounts of exchangeable potassium, an increase in growth followed the potassium increments. The nitrogen fixation within the plant decreased, however, with larger amounts of exchangeable potassium. The percentage and the total amount of calcium also decreased decidedly, while the percentage and the total of potassium increased equally noticeably. The data, as given in Table 2 for these elements, show the failure of the plants to use the calcium, whereas their use of the potassium is a distinct feature. These relations and the decreasing concentration of nitrogen are given graphically in Figure 6.

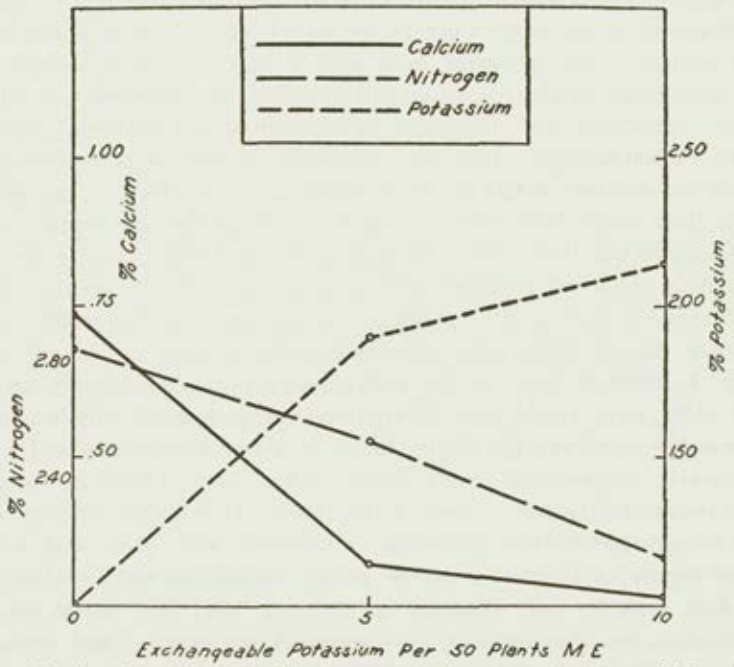

FIG. 6. Reciprocal relation of calcium and potassium concentrations within soybean plants associated with declining nitrogen concentration as the result of increasing potassium provided.

Since the clays were all completely saturated, the introduction of the increasing amounts of potassium along with the constant amounts of calcium and magnesium, lessened the degree of calcium saturation from 66, to 50 and 40 per cent, respectively. That this decreasing saturation is not the sole reason for calcium reduction in the crop is indicated by the use of the calcium at a higher percentage efficiency in these trials than was the case in the previous saturation studies in the absence of magnesium and potassium. It suggests, rather, that the calcium was replaced by potassium. When the calcium concentration is represented by potassium equivalents and these are

added to the potassium units as concentration within the plants, the results give almost a constant equivalent for the concentration of these two mineral elements for the three treatments. This suggests a calcium replacement by the potassium. With this replacement came a lowered nitrogen concentration and a lower nitrogen fixing activity.

POSSIBLE CALCIUM-POTASSIUM RATIO IN SOIL DEVELOPMENT SUGGESTED BY NITROGEN FIXATION STUDIES

As a theoretical consideration, it may be of interest to note the indication in these results that with a narrow potassium-calcium ratio, the concentration of nitrogen in the soybeans was high, or, as a microbial ration in the form of green manure, these soybeans have also a narrow carbon-nitrogen ratio. With a widening of the potassium-calcium ratio, the nitrogen concentration decreased decidedly, and such plants would represent a bacterial ration with a wider carbon-nitrogen ratio. On soils with the narrow potassium-calcium ratio, green manures would fix more nitrogen to be added to the soil, and this in turn would hold more carbon to build up the soil organic matter. This raises the question whether we might not expect soils in partly developed, or relatively immature, condition with large amounts of exchangeable calcium, and therefore a narrow ratio of potassium-calcium in exchangeable form, to encourage nitrogen fixing plant growths in order to build up the soil organic matter? Conversely then, as the exchangeable potassium-calcium ratio becomes wider, as is true in more highly developed or leached soils, would it be unreasonable to expect the plants to be of a less nitrogenous and a more carbonaceous composition? Such plants are less likely to build up an organic matter reserve in the soil because of the absence of a liberal nitrogen supply where no nitrogen fixation can occur. Chernozem soils, then, with a liberal calcium supply, or possibly a narrow potassium-calcium ratio, probably owe their dark color to their efficiency as nitrogen fixers and carbon retainers. Podzolization, on the contrary, represents calcium removal and consequent reduction in the total calcium to a low level and a wider potassium-calcium ratio to foster nonleguminous plants, even to those of distinctly woody nature. Such reasoning may not be amiss as applied to an ecological series of plants, when one considers that within a single kind of plant, as the soybean, the shift in the potassium-calcium ratio can change the plant from a proteinaceous to a carbonaceous producer.

SUMMARY

By means of studies using electrodialyzed colloidal clay, partly and completely saturated by different nutrient ions, the role of the soil factors in nitrogen fixation by legumes may be more clearly understood. Such studies lead to the following beliefs:

As the soil texture becomes heavier, legumes may be grown more successfully because of the larger supply of exchangeable bases, particularly calcium, offered by the increased clay content, than is at their disposal in soils of lighter texture, or with less clay of the same mineral nature.

The degree of acidity is not of direct significance in nitrogen fixation; but rather its more common reciprocal, the degree of calcium saturation, plays the significant role.

Nitrogen fixation by legumes is closely related to the amount of exchangeable calcium which the crop can take from the soil. The amount so taken is not necessarily related to the total supply: it may be only a small and variable portion of this supply.

The efficiency with which the exchangeable calcium is taken is related to the degree of calcium saturation. The efficiency increases decidedly as the degree of saturation increases. No nitrogen fixation occurs in Beidellite clay, unless the calcium saturation exceeds 50 per cent and the clay carries only adsorbed inorganic ions.

When the larger organic cations are adsorbed on colloidal clay, the accompanying inorganic cations are more effectively used by the plants. This suggests a possible explanation for effective nitrogen fixation by legumes on soils of higher organic matter content, even under significant degrees of acidity.

Calcium plays a possible role in the assimilation into the growing plant of the seed phosphorus, which may even be lost to the soil by soybeans, for example, at low calcium levels. No nitrogen fixation is possible when such loss occurs.

Exchangeable magnesium is of significance in nitrogen fixation possibly indirectly through its influence in increasing the effectiveness of calcium.

Exchangeable potassium of the soil plays a significant role in nitrogen fixation, but as larger amounts are taken in relation to the amount of calcium, the nitrogen fixation in relation to plant growth by legumes is reduced. The potassium may replace the calcium, thus resulting in a widening of the potassium-calcium ratio to the point where possibly the legume does not exercise its nitrogen-fixing ability.

The role of such a potassium-calcium ratio may possibly be helpful in understanding the relation of the organic matter levels in different soils in relation to their degree of calcium depletion, or to their stage in soil development.

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OBSERVATIONS ON THE HYPOCHLORITE OXIDATION OF DECOMPOSED PLANT RESIDUES*

ARTHUR GEOFFREY NORMAN

Iowa State College, Ames, Iowa, U. S. A.

THE processes involved in the biological decomposition of plant materials, whether in the soil or on its surface, bring about the removal of individual constituents at unequal rates depending on their relative availabilities to the active flora. Concurrently with the degradative changes, there is some synthesis of microbial tissue on the part of the organisms concerned. Under aerobic conditions when fungi may be active, and with an ample supply of nitrogen, the synthesized material may form 20 to 30 per cent of the decomposing residues. The presence of microbial material is made evident by the increase in organic nitrogen due to the formation of protein and chitinous compounds. Under anaerobic or waterlogged conditions, or when the development of fungi is suppressed, the amount of synthesized material is very small.

It is now generally accepted that of the major plant constituents, lignin is, under ordinary conditions, the least readily available, and that, as a result, the lignin content of decomposing material steadily rises. The tendency, therefore, is for extensively decomposed residues to contain a high percentage of lignin, and if formed under aerobic conditions then also an organic nitrogen content higher than that of the parent material. In table 1 are given analyses of some naturally decomposed materials, demonstrating the accumulation of lignin.

Ample evidence has been adduced to show that many of the properties of humified materials and soil humus can be accounted for on the assumption that residual lignin and synthesized nitrogenous complexes are the active components, the former, in general, far outweighing the latter in importance. For example, it was shown that from 65-97 per cent of the exchange capacities of aerobically decomposed residues of four common materials was contributed by the lignin,[1] and similarly it has been found that the buffer activity of humified materials is largely centered in the lignin fraction. Residual lignin is not necessarily homogeneous or unchanged, but may be a mixture of

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TABLE 1

LIGNIN AND APPARENT PROTEIN (N × 6.25) CONTENTS OF PEAT AND FOREST LITTER SAMPLES

	Lignin	Protein
	per cent	*per cent*
Sedgemoor peat	69.0	5.8
Lewis peat	54.7	13.0
Aboyne oak LL	35.7	8.6
Aboyne oak F	42.0	12.3
Ballochbuie pine LL	33.9	3.1
Ballochbuie pine F	50.0	7.7
Ballochbuie pine H	62.2	8.6

unattached and modified lignin. The nitrogenous component is not necessarily protein but may also include residues of a chitinous type. This newer concept of the humus treats it not as a substance of definite chemical composition but as a product progressively changing as decomposition proceeds. It would be desirable, therefore, to have some simple means of determining, or obtaining a measure of the lignin or lignin-derived fraction of decomposing residues that could also be applied to soils. If this should include some contribution by the nitrogenous complexes, then the result could be taken, in a sense, as an index of humification.

Most of the procedures suggested for the fractionation and study of decomposed residues and soil organic matter, such as those of Keppeler[4] and Waksman and Stevens[10] involve acid treatments and take advantage of the great resistance to strong acids possessed by lignin. In contrast to its inertness to acid and alkaline reagents, isolated lignin is known to be peculiarly susceptible to oxidants, the molecule in most cases undergoing extensive fission, with the result that after treatment only comparatively simple acidic products are to be found. Decomposed plant residues are well known to be far more readily oxidized than the parent plant materials, though it has probably not been widely recognized that this is primarily due to accumulation and liberation of lignin. Hot hydrogen peroxide has often been employed in the removal of organic matter from soils, since it has been shown[6] that the presence of soil catalyzes the oxidation of even such resistant polysaccharides as cellulose. Estimates of the extent of "humification" of soil have been made by weighing the residue after treatment, but such estimates have no real significance, since undecomposed materials are partly oxidized by this reagent. More vigorous oxidants, such as permanganate and chromic acid, have frequently been used for the oxidation of humus and humus extracts, but these methods virtually amount to the incomplete determination of total carbon. Sodium hypobromite

has been employed in the removal of organic matter from soils prior to mechanical analysis,[8] and sodium hypochlorite has been shown to react with soils of high organic content.[5] Sibirsky[7] treated various soils with a large excess of hypochlorite in relatively high concentration and determined the residue after treatment, the difference being regarded as humified material. Isolated lignin is known to be extensively oxidized by hypochlorous acid in low concentrations,[2] and this reagent also reacts rapidly with nitrogenous substances containing amino groups. In view of the ease with which such oxidations can be followed titrimetrically, a study has been made of the hypochlorite oxidation of decomposing materials and of lignin preparations isolated from them.

OXIDATION OF ISOLATED LIGNIN PREPARATIONS BY HYPOCHLORITE

The structure of lignin is as yet incompletely understood. Phenolic degradation products may be obtained, but in poor yields. Stable methoxyl groups are present, and in addition at least an equal number of hydroxyl groups. In view of observations made on the hypochlorite oxidation of phenol and salicylic acid in connection with studies on phenylalanine and tyrosine, the hydroxyl groups might be expected to be points of susceptibility to oxidative attack. Hibbert and Taylor[2] found the reaction between lignin and hypochlorous acid to be actively catalyzed by hydrochloric acid and ascribed about a third of the uptake during the first hour or two to the oxidation of a phenolic residue. In our experiments the oxidation was carried out in high dilution, in general 50 mgm. of acid lignin being treated in a total volume of 100 ml. with sodium hypochlorite supplying 200-250 mgm. available chlorine. Under such circumstances, either in faintly or in strongly alkaline solution, or when acidified, lignin is extensively oxidized. The reaction is most rapid in the first few hours but continues for days, no final end-point being reached. Under strongly alkaline conditions (1 per cent NaOH) the rate of change is slow after 2 or 3 days, but in such circumstances the uptake is only a little over half of that in almost neutral solution.

For general purposes the almost neutral conditions provided by unadjusted hypochlorite solutions seem most useful. In the accompanying tables ordinarily only the figures for chlorine utilized in 2 hours and at the end of 3 or 5 days are given, though intermediate values were obtained. The rate and extent of oxidation of lignins from different sources are not identical, though the shapes of the curves are very similar (table 2). Alkali lignin preparations are oxidized less extensively than acid lignins in equal times. The chlorine ultimately utilized in 5 days under acid conditions is between 2 and 3 mgm. per mgm. lignin, in most cases being about 2.5 mgm. Several workers have suggested that the basal molecular unit weight of lignin is of the order

TABLE 2

OXIDATION OF LIGNIN PREPARATIONS BY HYPOCHLORITE UNDER VARIOUS CONDITIONS

Uptake expressed as mgm. Cl used per mgm. lignin

Lignin source	Neutral*			Acid†			Alkaline‡		
	2 hrs.	3 days	5 days	2 hrs.	3 days	5 days	2 hrs.	3 days	5 days
Oat straw (acid lignin)...	0.7	1.49	1.91	0.8	2.05	2.32	0.5	1.08	1.05
Oak (acid lignin).....	1.0	2.09	2.41	1.1	2.37	2.61	0.8	1.46	1.47
Barley straw (acid lignin).	0.8	2.37	2.70	0.8	2.09	2.37	0.5	1.18	1.18
Barley straw (alkali lignin)	0.7	2.12	2.48	0.7	1.56	2.47	0.6	1.24	1.35
Pine (acid lignin).....	0.6	2.21	2.65	0.9	2.45	2.73	0.6	1.25	1.57
Pine (alkali lignin).....	0.2	1.42	1.63	0.4	1.54	1.88	0.4	0.88	1.11

* Final alkalinity 0.003 *N* approx.
† Final acidity 0.002 *N* approx.
‡ Final alkalinity 0.25 *N* approx.

of 900. On this basis the uptake of 2.5 mgm. Cl per mgm. is equivalent to about 32 oxygen atoms, which is only about one-third of that necessary for complete oxidation to CO_2 and water. The reaction is, however, not complete at 5 days and continues slowly for a long period, but the instability of the hypochlorite in the control makes it impossible to rely on extended experiments. Both rate and extent of oxidation are affected by the relative concentrations of the reactants and by the ratio of hypochlorite to lignin.

OXIDATION OF PROTEINS BY HYPOCHLORITE

As is well known, proteins react extensively with hypochlorite if excess of chlorine is present. The uptake is not appreciably affected by the alkalinity or acidity of the solution and ultimately reaches about 3 mgm. Cl per mgm. protein, equivalent approximately to 10 atoms of Cl per atom of nitrogen (table 3).

TABLE 3

OXIDATION OF PROTEINS BY HYPOCHLORITE UNDER VARIOUS CONDITIONS

Uptake expressed as mgm. Cl used per mgm. protein

	Percentage N	Cl/protein = 4/1			Cl/protein = 8/1		
		2 hrs.	2 days	4 days	2 hrs.	2 days	4 days
Casein.....	12.85	1.14	2.62	2.88	1.26	2.98	3.24
Albumin.....	12.3	1.58	2.68	3.00	1.56	2.96	3.12

It will be noted that the amount of chlorine used by proteins is of the same order as that used by lignin, and this must be taken into account in the consideration of values from aerobically decomposed residues.

OXIDATION OF DECOMPOSED RESIDUES AND ACID LIGNIN PREPARATIONS THEREFROM

The hypochlorite oxidation of a number of plant residues decomposed naturally and under controlled laboratory conditions was studied. Acid lignin preparations were also obtained from some, and these were similarly determined (table 4). Despite the precautions of pretreatment, the nitrogen con-

TABLE 4

OXIDATION OF DECOMPOSED RESIDUES AND ACID LIGNIN PREPARATIONS THEREFROM

Chlorine expressed as mgm. Cl per mgm. sample

Material	Lignin	Total N	Cl uptake by residue		Cl uptake by lignin		N in lignin
			2 hrs.	5 days	2 hrs.	5 days	
	per cent	*per cent*					*per cent*
Sedgemoor peat	69.0	0.93	1.03	2.37	0.87	3.05	0.92
Lewis peat	54.7	2.08	0.48	1.65	0.85	2.09	2.06
Aboyne oak LL	35.7	1.37	0.40	0.97	....	....	....
Aboyne oak F	42.0	1.97	0.41	0.98	....	....	....
Ballochbuie pine LL	33.9	0.49	0.36	0.76	0.96	2.46	0.85
Ballochbuie pine F	50.0	1.24	0.53	1.22	0.94	2.53	1.36
Ballochbuie pine H	62.2	1.38	0.62	1.55	1.05	2.59	1.50
Rye straw rotted 3 years	44.8	3.08	0.51	1.72	0.96	2.89	2.80
Oat straw	13.7	0.38	0.21	0.70	0.65	1.82	1.02
Oat straw rotted 1 year (aerobic conditions)	31.1	3.28	0.27	1.13	0.67	1.82	2.81
Oat straw rotted 1 year (anaerobic conditions)	21.8	0.40	0.11	0.44	0.66	1.54	1.09
Oat straw rotted 1 year (waterlogged conditions)	23.4	0.41	0.10	0.40	0.68	1.56	1.01

tent of such preparations from decomposed residues is always distinctly higher than those from the parent materials, as has been pointed out elsewhere.[1]

An examination of the data in table 4 shows that in the extensively decomposed residues, such as Sedgemoor peat and the pine litter series, in which lignin

has accumulated to a considerable extent and of which the nitrogen content is not high, the activity of the lignin accounts almost completely for the activity of the whole residue. This assumes that the susceptibility of the lignin to oxidation is not markedly increased or diminished by the process of isolation. With higher contents of nitrogen, in the rotted rye straw and Lewis peat, for example, as might be expected, the divergence between the total value and that due to the lignin increases.

The presence of nitrogen groupings condensed with the lignin in the preparations from residues of high nitrogen content is a confusing factor, since the condition of the nitrogen is not then the same as in natural residues. By the isolation of lignin from straw in the presence of added protein, lignin preparations of high nitrogen content were obtained, the apparent susceptibility of which to oxidation was greater than that of the pure preparation (table 5).

TABLE 5

OXIDATION OF WHEAT STRAW LIGNIN PREPARATIONS OBTAINED IN THE PRESENCE OF ADDED PROTEIN

	N	Chlorine uptake per mgm.		
		2 hrs.	3 days	5 days
	per cent	*mgm.*	*mgm.*	*mgm.*
Wheat straw lignin	0.51	0.66	1.39	1.78
Wheat straw lignin (casein added)	2.85	0.66	1.63	1.91
Wheat straw lignin (albumin added)	2.97	0.72	1.82	2.31

It is therefore to be expected that lignin preparations obtained from aerobically decomposing residues in which the nitrogen content is increasing will show an apparent increase in activity, which must be ascribed to the presence of condensed nitrogen and not to any possible chemical change in the lignin as a result of decomposition processes. No indication was obtained of any increase in susceptibility to oxidation as a result of chemical change in the molecule, though this does not preclude the possibility of minor changes in substituent groupings. The best example is provided by the three lignin preparations from the successive layers of the pine forest litter in which the nitrogen content was not high, since these were very similar in reactivity (table 4). Information along the same line was sought by examining the residues of the aerobic decomposition of oat straw (table 6). The lignin preparations obtained increased progressively in nitrogen content, and the apparent increases in activity that occurred were no larger than could be accounted for on this basis. Under waterlogged or fully anaerobic conditions, when no immobilization of nitrogen takes place and when in consequence the purity of the lignin preparations is not affected, there was an apparent reduc-

TABLE 6

OXIDATION OF OAT STRAW ROTTED AEROBICALLY, AND ACID LIGNIN PREPARATIONS THEREFROM

Chlorine uptake expressed as mgm. Cl per mgm. sample

Period of decomposition	Residue	Total N	Lignin	N in lignin	Cl uptake by residue		Cl uptake by lignin	
					2 hrs.	5 days	2 hrs.	5 days
	per cent	*per cent*	*per cent*	*per cent*				
....	100	0.4	13.7	1.0	0.21	0.70	0.65	1.82
1 week.....	85.7	1.3	18.6	1.2	0.26	0.78	0.50	1.79
2 weeks.....	71.6	1.7	22.9	2.2	0.33	0.99	0.52	1.88
6 weeks.....	55.6	2.0	28.6	...	0.34	1.06	....	....
3 months....	48.2	2.3	30.4	2.5	0.40	1.17	0.64	2.13
6 months....	42.7	2.6	28.9	2.8	0.41	1.29	0.73	2.30
9 months....	40.7	2.8	28.3	3.4	0.47	1.30	0.73	2.36
12 months....	38.0	2.9	23.1	2.7	0.41	1.25	0.80	2.48
18 months....	33.5	3.1	26.4	3.0	0.41	1.21	0.60	2.34

tion in susceptibility to oxidation (table 4). This change is being studied further. Residues and lignin preparations alike gave appreciably lower results than did the parent straw.

The uptakes of chlorine by proteins and lignin preparations are individually of much the same order, though the former react more quickly. Since it has been suggested that the lignin and protein components of plant residues are combined or associated into some form of complex from which the nitrogen is biologically less available than that from the original components, the behavior of such mixtures towards oxidation by hypochlorite was determined. To oak lignin suspended in alkali a solution of albumin was added, and the mixture was precipitated at pH 4.5 according to the method of Hobson and Page.[3] A similar mixture with casein was precipitated in the presence of calcium chloride at pH 7.0 according to the procedure of Waksman and Iyer.[9] The oxidation of these materials is shown in table 7. It is apparent that one of the results of mutual precipitation is that the complex is initially less readily oxidized by hypochlorite, though over a longer period this difference is not maintained. This observation was verified by the preparation of a series of lignin-protein mixtures, all from oat straw lignin and casein (table 8). The sums of the values calculated for the lignin and casein present were greater than the experimental values for the first 2 hours, but later the oxidation proceeded as though the components were oxidized independently. It is to be inferred from these observations that the curve representing the oxidation of a decomposed plant residue high in protein is likely to be flatter in the initial stages than that of the lignin isolated from it, and

TABLE 7

OXIDATION OF OAK LIGNIN-PROTEIN MIXTURES

Substance	Total N	Chlorine used per mgm. sample		
		½ hr.	2 hrs.	3 days
	per cent	*mgm.*	*mgm.*	*mgm.*
Oak lignin	0.2	0.78	1.12	2.09
Oak lignin + casein	1.5	0.33	0.62	2.09
Oak lignin + albumin	3.2	0.43	0.79	2.37
Casein	12.9	0.70	1.22	3.24
Albumin	12.3	0.93	1.48	3.12

indications of this have been obtained experimentally on the rye straw residue and the Lewis peat. Support is also provided for the view that actual com-

TABLE 8

OXIDATION OF PRECIPITATED OAT STRAW LIGNIN-CASEIN MIXTURES

Casein content	Cl used per mgm. sample					
	½ hr.		2 hrs.		5 days	
	Found	Calc.	Found	Calc.	Found	Calc.
per cent	*mgm.*	*mgm.*	*mgm.*	*mgm.*	*mgm.*	*mgm.*
69.1	0.58	0.64	0.92	1.04	2.61	2.63
58.5	0.58	0.62	0.88	0.98	2.57	2.51
41.0	0.56	0.58	0.85	0.89	2.51	2.30
25.4	0.42	0.55	0.72	0.80	2.31	2.12
18.4	0.48	0.54	0.68	0.76	2.06	2.03
14.0	0.48	0.53	0.68	0.73	1.99	1.99
....	0.50	....	0.65	....	1.82	....

bination occurs when lignin and protein are precipitated together in this manner.

DISCUSSION AND SUMMARY*

Aerobic decomposition processes result in the progressive accumulation of lignin and nitrogenous complexes, which are particularly susceptible to oxida-

* Much of the work described was carried out by Marian Norman at the Rothamsted Experimental Station.

tion by hypochlorite. No indication has been obtained that the increase in chlorine uptake on the part of aerobically decomposed materials is due to anything more than the accumulation of these components; that is to say, there is no clear evidence for a change in reactivity on the part of the lignin. This reaction may therefore be employed in following the progress of decomposition or comparing residues obtained from the same parent substance and is particularly applicable to such materials as the successive layers of raw humus on the forest floor forming the A_0 horizon, or to layers in a peat profile. On the other hand, hypochlorite oxidation would not seem to be very suitable for the comparison of residues of entirely different sources and types, mainly because of the differences in reactivity and behavior of the lignin which they contain. As the reaction is not a completed one, the values obtained are not absolute. Comparisons should always be made at equal dilutions and with excess of hypochlorite, approximately the same initial ratio of chlorine to oxidizable material being maintained. No attempt should be made to interpret the results as indicating the amount of "humified organic matter" present or the "degree of humification," but the increase in reactivity may be taken as a measure of the accumulation of those components, lignin and nitrogenous, which markedly affect many of the properties of decomposed residues.

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EFFECTS OF ORGANIC AMENDMENTS UPON THE MICROFLORA OF THE RHIZOSPHERE OF COTTON AND WHEAT*

FRANCIS E. CLARK and CHARLES THOM

Bureau of Plant Industry, U. S. Department of Agriculture, Washington, D. C., U. S. A.

EFFECTS of decomposing organic matter upon total populations of the soil or upon the presence and activities of individual species have been studied extensively from the standpoint of fertilizing values. Although Pammel as early as 1888[7, 8] recognized the efficacy of stable manure in checking cotton root rot, it was only following more recent demonstrations of successful manurial controls, such as those reported for root rot of cotton by King,[4, 5] for potato scab,[6, 10] for take-all of wheat,[3] and for flax wilt,[1] that control of disease-producing fungi was assumed by various authors to result from changes or activities in the general micropopulation induced by manure. It becomes essential, therefore, to analyze the microbiological effects of soil treatments from the point of view of soil hygiene, to seek for useful microbic responses and to furnish if possible the basis of recommendations for the employment of particular procedures. Accordingly, the present studies were undertaken to investigate microbiological effects of organic matter in the rhizosphere of cotton and wheat under conditions for which control of root rots of these crops had been reported.

WHEAT RHIZOSPHERE

Investigations on the rhizosphere of wheat† were made under greenhouse conditions. A heavy loam soil, obtained from a field near Delavan, Kansas, naturally infested with the take-all fungus, *Ophiobolus graminis* Sacc., was employed under the following conditions: untreated; fertilized with chicken manure (one part in six by volume); fertilized with chopped green alfalfa

* Investigations conducted cooperatively by the Divisions of Soil Microbiology, Cereal Crops and Diseases, and Cotton and other Fibre Crops and Diseases, Bureau of Plant Industry, and the Kansas Agricultural Experiment Station. Contribution No. 180 of the Department of Bacteriology, Kansas State College of Agriculture and Applied Science.

† We wish to acknowledge our indebtedness to Hurley Fellows for his cooperation and assistance throughout the course of this work.

hay (one part in six by volume); and steam-sterilized, and therefore *Ophiobolus*-free. Potted soils were immediately planted to wheat. In the collection of wheat roots for study, the entire volume of soil in a container was removed; careful shaking was then employed to obtain the root system as nearly free of soil as possible. The root systems thus collected were suspended in nine times their own weight of sterile water and shaken vigorously for 10 minutes with glass beads before subsequent dilutions were made. Portions of soil free of readily observable root fragments were also obtained for study. The details of these experiments have been discussed elsewhere.[2]

Fertilizations effective in greenhouse control of take-all exerted the customary stimulation of soil microorganisms; increases were noted in numbers of total bacteria, filamentous fungi, actinomyces, and various minor groups of bacteria. The comparative increases for total bacteria following chicken manurial or green-alfalfa treatments of *Ophiobolus*-infested soil cropped to wheat are shown in Table 1.

TABLE 1

COMPARATIVE BACTERIAL POPULATIONS IN GREENHOUSE SOILS

Time following application of treatment.....days	3	8	14	24	43	60
	Bacterial numbers*					
Chicken manure added....	1,351	1,551	1,170	739	408	310
Chopped alfalfa added....	618	275	160	93	122	165
Untreated.....	27	56	85	44	53	38

* Numbers for 1/1,000,000 dilution of 1 gm. of air-dry soil.

Microfloras of wheat roots grown in such soils were investigated. For total bacteria, the most important numerical group associated with plant roots, outstanding quantitative differences due to soil treatment were not apparent for nearly two months; by that time, severe take-all damage in untreated soil, and variations in plant responses in treated soils, were also apparent. Prior to the time that plant differences became obvious, micropopulations associated with roots of young wheat rose from comparatively low initial levels to levels in excess of one billion with increasing age of roots, and independence from soil populations was repeatedly indicated. To exemplify, root micropopulations determined for wheat 43 days old, grown under the soil conditions listed in Table 1 were 1,150, 1,165, and 1,123 millions, from chicken-manured, alfalfa-treated, and untreated soils, respectively.

When chicken-manured, steam-sterilized, and untreated soils were placed in 4-inch horizontal layers in single containers 12 inches deep, with various al-

terations in the positions of soils, the manured soil invariably had the highest total bacterial population, regardless of its position in a container. The highest root micropopulations, however, did not always parallel the highest soil populations, but were generally found associated with the root segments occurring in the top layer, regardless of the soil layered in the top position. Although for numbers of total bacteria on roots, soil treatment did not appear so important as position in container, there was nevertheless some influence of soil treatment. This influence was particularly pronounced for certain groups. Manured soil layers invariably had the greatest numbers of aerobic spores, regardless of position in container. Root segments from manured soil invariably possessed increased numbers of aerobic spores. Similar relationships were irregularly noted between root surface and soil populations of actinomyces and filamentous fungi. As parallel relationships were not noted in studies on cotton, it was considered probable, in dealing with the fibrous root system of wheat, that soil particles, and therefore soil micropopulation effects, were not readily eliminated from root material collected. Observations obtained on soil and root samples collected from pots identical except for differences in moisture content at time of collection of samples indicated such a possibility. With increasing moisture content at sampling, and therefore increased difficulty in obtaining root segments relatively free of soil, the ratio, root numbers/soil numbers, for spores of aerobic bacilli approached unity for moist soils but decreased to smaller fractions of unity with decreased moisture content at sampling. On the contrary, for total bacteria, the ratio, root numbers/soil numbers, showed the larger multiples of unity at decreased moisture content at sampling. It was concluded that the root surface bacterial flora, intimately associated with root surfaces and depending upon nutrient materials coming from roots, was not readily affected by soil amendments or by variations in the microflora of the adjacent soil.

COTTON RHIZOSPHERE

Investigations on the rhizosphere of cotton* were made during the summer months on field plats of the U. S. Cotton Field Station, Greenville, Texas. Plats chosen for study had received applications of some organic material, as stable manure, sorghum bundles, straw, or cotton seed meal or hulls, prior to or at the time of planting. In the collection of cotton root material, the major portions of from three to seven separate root systems within a plat were excavated. The surface layers were then peeled or scraped from the roots and partially triturated with mortar and pestle. Only healthy plants were chosen for study; care was taken to avoid collecting roots from, or in the

* We wish to acknowledge our indebtedness to Dalton R. Hooton, acting superintendent of the U. S. Cotton Field Station, Greenville, Texas, and to others of the station staff for assistance and cooperation throughout the course of this work.

immediate vicinity of, diseased plants, in order that possible effects of soil treatments would not be obscured by the microbic activity resulting from root decomposition.

Determinations were made of micropopulations associated with root surfaces of cotton in untreated plats and of cotton in experimental plats having received deep trench applications of stable manure, sorghum bundles, or sorghum bundles plus ammonium sulfate (500 pounds per acre). Total bacterial populations associated with roots from such soils are shown in Table 2. Outstanding differences in root numbers due to soil treatment were not apparent.

TABLE 2

TOTAL BACTERIA* ASSOCIATED WITH ROOT SURFACES OF COTTON GROWN UNDER VARIOUS SOIL CONDITIONS

Field treatment	July 13	July 27	August 10	August 24
Stable manure.....	22.3	57.3	246.0	57.7
Sorghum bundles....	45.7	69.3	157.0	53.0
Sorghum plus ammonium sulphate.....	39.7	33.5	210.0	41.0
Untreated check.....	18.0	67.3	213.0	51.0

* Numbers for 1/1,000,000 dilution of 1 gm. of root surface material.

Excepting extremely high micropopulations (2,000 to 3,000 million per gram) localized in remnants of organic materials applied, the general soil populations for field treatments shown in Table 2 did not vary greatly during the dry summer season. But even in the presence of apparent soil population differences in certain barrier experiments, root micropopulations remained comparable, as illustrated by the occurrence of root micropopulations of 62.0, 66.0, and 60.7 millions in barrier soils showing bacterial populations of 92.8, 44.3, and 8.5 millions, respectively.

For certain blocks of one field experiment, in which cottonseed meal or hulls or both together had been applied at planting time, at rates of 2,000 pounds per acre for meal and 1,000 pounds per acre for hulls, the organic material applied had been inoculated with *Bacillus subtilis* 24 hours before placement in the soil. Table 3 shows soil and root bacterial populations determined on September 7, as well as the abundance of the inoculated genus *Bacillus*.

From cultural and tinctorial comparisons of 800 cultures isolated from root surfaces of cotton grown under the field conditions shown in Table 3 or from such soils themselves, pronounced differences were not apparent between isolates from roots of variously treated cotton plants, although differences were

TABLE 3

BACTERIAL POPULATIONS FOLLOWING APPLICATIONS OF ORGANIC MATERIAL INOCULATED WITH *Bacillus Subtilis*

Soil condition	Total Bacteria*		Genus *Bacillus**	
	Soil	Root surfaces	Soil	Root surfaces
Untreated check soil.....	12.77	89.7	2.13	.020
Cottonseed hulls.....	45.27	73.0	1.57	.076
Hulls and *B. subtilis*.....	58.97	49.0	2.37	.143
Cottonseed hulls and meal	106.6	54.0	38.0	.196
Hulls, meal, *B. subtilis*...	143.9	72.0	59.2	.323
Cottonseed meal only....	148.5	73.0	39.7	.564
Meal and *B. subtilis*.....	100.0	45.0	35.3	.740

*Numbers at 1/1,000,000 dilution of 1 gm. of soil or root surface material.

readily noted between isolates from soil and isolates from roots. Distinction between the soil flora and the root flora is shown in the results given for the inoculation experiment (Table 3). High numbers (35 to 59 millions) of aerobic bacilli were encountered following application of cottonseed meal. In soils receiving cottonseed meal heavily inoculated with *B. subtilis*, the inoculated organism was noted as predominant; in uninoculated soils, *B. megatherium* was predominant. Regardless of variations in numbers or in predominant types of *Bacillus* in the soils, the numbers of this group associated with root surfaces were in no instance increased to as many as one million per gram of root material.

DISCUSSION

It is apparent that although organic amendments produce striking changes in the micropopulations of the earthy mass, comparatively stable micropopulations are associated with the crop roots themselves. The decomposition periods following manurial applications are characterized by high microbial numbers in the soil, but root micropopulations at the time of or subsequent to the periods of decomposition are relatively independent of soil variations. Even when a particular species (*B. subtitlis*) was established in the soil by combined inoculation and fertilization procedures, the incidence of this species or of its genus upon the root surfaces themselves was not materially changed. This comparative stability of the root surface microflora makes questionable the possibility of protecting such surfaces from parasitic invasions by the inoculation of common saprophytic microorganisms of the soil, at least insofar as such protection may be obtained on a practical scale in the field. Although

it is well known that experimental controls of root-rotting parasites have been obtained by the inoculation of diseased soil with saprophytic cultures or filtrates thereof, it is questionable whether simple inoculation effects have always been obtained. The fact that the filtrates themselves frequently gave some measure of control suggests that the controlling principle has in some instances been applied rather than originated under the environmental conditions of the soil. In the evaluation of the practicability of direct application of cultures or filtrates, it may be recalled that Potter[9] demonstrated the control of a *Pseudomonas destructans* rot of turnips by the application of autoclaved cultures of the causative microorganism itself. Once a root-rotting parasite reaches the roots, factors of host resistance or factors of virulence of the causal organism are probably more important than microbic competition or microbic by-products and activities on root surfaces, or at least more important than microbic factors which may be ascribed directly to manurial treatments.

The stability of the root surface microflora following organic amendments, in contrast to the responsiveness of the soil microflora, makes it plausible to assume that the activities of the soil microflora are of primary importance, insofar as microbiological factors are concerned, in the manurial control of root-rotting parasites. Whether the possible sanitation effects are accomplished in a nonspecific and quantitative manner, or whether they result from the interactions of but a few species offers a field for extensive investigation; thus far, antagonism due to a particular species has not been found sufficient to account for the results obtained.

Whether soil sanitation is accomplished during the course of the growing season or is limited to a relatively short period of bacterial activity prior to that period in which plant losses generally become obvious is probably determined largely by moisture, temperature, and aeration conditions, as well as by the kinds or amounts of organic materials applied. Under the conditions of the Greenville experiments, the critical microbiological period would appear to be prior to the summer months, for during these months, soil activity following treatment is not materially different from that in untreated soil, except in the trenched remnants of the organic material applied. Such remnants exist as islands of high activity, with populations of billions, but such activity does not affect the surrounding soil to distances greater than fractions of an inch. As cotton roots do not enter such islands of activity, but are prone to detour around them, the importance of the microbic activity in such islands during the summer in controlling *Phymatotrichum* is questionable. It is, of course, possible that the deflection of cotton roots by trenched organic material, or the encouragement of a more superficially branched root system may be of importance quite aside from the question of microbic antagonism.

Although the present investigations have indicated that seed or root

inoculation procedures in the field may be of questionable value in controlling root rots, particularly if common soil microorganisms not adapted to the root surfaces are employed, possibilities for exploitation still remain. The importance of the amount or kind of organic material and of accessory fertilizing materials, the time of application, and the period of activity allowed remains for extended study.

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THE METHOD OF PROXIMATE ANALYSIS AND ITS APPLICATION TO THE STUDY OF PLANT RESIDUES, COMPOSTS, AND HUMUS FORMATIONS*

SELMAN A. WAKSMAN

New Jersey Agricultural Experimental Station, New Brunswick, N. J., U. S. A.

IT HAS long been recognized that a better understanding of the processes involved in the decomposition of plant residues and a better knowledge of the origin and nature of humus can be obtained if the chemical composition of the plant materials and their decomposition products are taken into consideration. Since the quantitative composition of a natural organic material depends largely upon the analytical procedures, it was important that the methods of analysis employed for these comparative purposes be essentially the same. The fact that the most extensive literature on the chemistry of humus, largely based upon the analysis of humic acid, humins, and similar bodies, did not throw sufficient light upon the nature and origin of humus was due principally to a lack of correlation between these procedures and the more chemically defined methods commonly used in plant analysis. Comparisons become even more difficult when the latter do not differentiate with sufficient clarity between the various chemical compounds. The methods of foodstuff analysis, for example, based largely upon arbitrary procedures[7] and difficult enough to interpret when applied to digestibility problems, become of little significance when applied to humus formations.[32]

Without attempting to review in detail the literature which led to the development of the proximate method of analysis of plant materials and their decomposition products, attention will be directed only to certain investigations which served as the basis for these methods. A summary will also be presented of other methods, proposed by different investigators, which serve similar purposes. The various applications of the proximate method to the study of the chemical composition of plant materials, of their decomposition products, and of humus formations in nature will be presented.

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THE PRINCIPLE UNDERLYING THE PROXIMATE METHOD OF ANALYSIS

Any proximate method of analysis of fresh plant materials, of their decomposition products, and of the resulting humus must be based upon the recognition, in quantitative terms, of certain well-defined chemical constituents of plants. It must be simple in nature. It must lend itself readily to mass analyses within a brief time. It must require only small quantities of material for analysis. It should further lend itself to various modifications, in order to confirm by more exact procedures the accuracy of the results thus obtained and widen our knowledge concerning the individual constituent groups. It need only be proximate in nature, accounting not for 100 per cent of the constituents but for, say, 90-95 per cent. The analyses should be carried out on as few samples as possible in order to avoid errors in overlapping.

By this method, one should be able to follow the fate of the various constituent groups through the different stages of disintegration of the plant residues and their gradual transformation into a brown- to dark-colored material, usually designated as "humus." One should be able further to determine the influence of environmental factors, soil conditions, and microbiological population upon the chemical and biological processes taking place in the decomposition of the material.

Among the various precursors of the method here under consideration, the following deserve mention. As far back as 1880, Sivers[51] proposed to separate the organic constituents of peat into five groups: 1. soluble in hot alcohol, 2. soluble in hot water, 3. soluble in hot 2 per cent HCl, 4. soluble in hot KOH solution, 5. insoluble residue. More recently, Keppeler[18] suggested measuring the degree of decomposition of peat by hydrolyzing it with mineral acids and distinguishing the hydrolyzable and nonhydrolyzable fractions; the relation between these fractions in peat is then compared with the corresponding relation in the plant material from which the peat originated. Kiesel and Semiganowsky[20] demonstrated that 100 per cent yield of true cellulose is obtained when a proper concentration of mineral acid is used, at low temperatures, followed by higher temperatures after the concentrated acid has been diluted with water. This procedure can be made to correspond to the method of obtaining "acid lignin." On the basis of these and other methods, Waksman and Tenney[95] proposed at first a system of analysis whereby different samples of material were employed for different determinations, the alkali-soluble fraction being also included. These two factors were responsible for certain confusing results.

This preliminary method was later modified to give the proximate system of analysis which is now in use[88, 93] and which forms the subject of this review. In this method, nearly all determinations are made on a single sample

of material, in duplicate or in triplicate. Separate samples are used for ash and total nitrogen determinations only. The following groups are usually taken into consideration in the proximate system of analysis:

1. Fatty and waxy materials, extracted by means of solvents. Ether or benzol-alcohol is usually employed for 8 to 24 hours. Other solvents, such as chloroform or alcohol, may also be used following ether. In some modifications of this method, a second ether extraction is made after the dilute acid hydrolysis, thus accounting for the combined fatty acids in addition to the neutral esters; in the case of certain humus formations, notably high-moor peats, the second extraction may increase considerably the yield of this group of organic constituents.

2. Water-soluble substances. The material is first extracted in cold water for 24 hours. This is usually followed by hot water extraction, at 100° C., for 1-3 hours. In many instances, only cold water extraction is employed, or hot water alone is used for a period of 3-5 hours. In some cases, especially in young plant material, it is desirable to determine also the water-soluble reducing sugars and total nitrogen.

3. Dilute acid hydrolyzable constituents. Here belong the starches, the polysaccharide hemicelluloses, and some of the polyuronides. This group of constituents is measured by determining the reducing sugars in the hydrolyzate and multiplying by 0.9. In view of the fact that polyuronides do not give 100 per cent reducing sugar, plant materials, like mosses and seaweeds, high in polyuronide hemicelluloses give considerably lower values than 100 per cent. This is due to the fact that the uronic acids are decarboxylated, on treatment with hot dilute mineral acids, and a variety of compounds, not all of which are reducing in nature. A correction can be made in this group of constituents by determining the uronic acid content of the material and making proper allowance.[31] In some cases,[66] the total organic matter in this fraction, based upon the soluble material or calculated from its carbon content, is reported.

4. Concentrated acid hydrolyzable constituents. Dilute acids, at normal pressure and at 100° C., do not attack true cellulose to any considerable extent.[20] The latter can be hydrolyzed by the use of concentrated acid in the cold, namely, 66-80 per cent sulfuric or 42 per cent hydrochloric acid or mixtures of these, for 1-6 hours. When the hydrolyzate is diluted with water and boiled, the cellulose becomes quantitatively converted to glucose. To ensure an accurate figure, this step must be preceded by dilute acid hydrolysis and removal of the products.

5. Lignin fraction. The preceding three treatments result in the removal of all the carbohydrates; the lignin, accompanied by some protein derivatives and a certain amount of mineral constituents, is left. When corrected for nitrogen and ash, a quantitative figure for lignin is obtained. Although this

method of lignin determination has frequently been criticized, it is as accurate as any of the others thus far proposed, the quantitative yields depending, of course, upon the method used.

6. Nitrogenous constituents. These are usually calculated from the total organic nitrogen in the material, using the factor 6.25. This fraction can be subdivided into water-soluble and water-insoluble groups. Occasionally, the nitrogen content of the lignin residue is of interest from the point of view of protein synthesis and protein-lignin relationships.

7. Mineral fraction. This is best determined by ashing an aliquot part of the plant material. Here again, it is possible to differentiate between the water-soluble and water-insoluble fractions.

The results obtained by the proximate method of analysis, as outlined above, usually account for 90 to 95 per cent of the total constituents of plant materials, stable manures, composts, peats, and similar organic matter formations. This method has a number of limitations, two of which may be pointed out here. Attention has already been directed to the first of these, the fact that, in the case of sphagnum plants and young sphagnum peats, marine algae and their decomposition products, cell substance of certain micro-organisms, and other materials high in polyuronides, this method may account for only 75-80 per cent of the constituents. The second important limitation of the method is that, in decomposed materials, most of the dark colored substances characteristic of humus are included with the lignin fraction. This fact was well recognized in developing the method, as is shown by the attempt to designate this fraction as "lignin and its derivatives" or "lignin-humus." Unquestionably, lignin undergoes various chemical changes in the process of decomposition of the plant materials and can no longer be recognized as native lignin. It is also possible that, during the decomposition, certain substances are produced either by chemical changes or by microbial activities, which have no relation to lignin and which are included in this fraction. Many of the criticisms and modifications of the proximate method as a whole were largely directed against this fraction. Some investigators attempted to separate the fraction into "lignin" and "humic bodies," by the use of various reagents, such as alkali solutions, acetyl bromide, hydrogen peroxide, potassium permanganate, which were believed to differentiate the humified or changed constituents from the unhumified or unaltered materials. These modifications are largely arbitrary, and as long as no definite chemical evidence can be brought to light to explain differences thus obtained, the group can be left as originally designated.

The criticisms directed against this system of proximate analysis have emphasized either the aforementioned two limitations or the arbitrariness with which some of the specific chemical constituents are determined. Other procedures, said to be more specific, were recommended for the determina-

tion of hemicelluloses (furfuraldehyde method), cellulose (hypochlorite-alkali sulfite method), and other constituents.[1]

Though the proximate system of analysis proved to be very convenient for the study of fresh plant materials, their decomposition products, composts, peats, and surface layers of forest soils, it could not be used directly for the study of humus in mineral soils. A simplified procedure for calculating the "lignin-humus" complex was suggested.[76, 83, 94] This takes into consideration the carbon and nitrogen content of the soil left after treatment with 80 per cent sulfuric acid. A definite correlation was found[58a] to exist between the color of the alkali extracts of soil and the lignin-humus complex.

The proximate method of analysis has also been variously modified. Tiurin[67] used cold acid in place of water, in order to remove the soil bases; he further modified the method by extracting the material with an alkali solution and considered the soluble organic matter as "humic acid" and the final unhydrolyzed residue as "humin-lignin." He also tried to account in further detail for some of the various constituents of certain groups, especially the carbohydrates and the water-soluble fraction. In order to account for the uronic acid compounds in sphagnum plants and peats, Schmako[46] suggested that the dilute acid treatment be followed with dilute alkali extraction. The organic acids brought into solution by the acid and alkali treatments are precipitated as copper salts and reported as a separate fraction.

In some cases, the proximate method has been modified by combining two or more fractions, as in the analysis of mineral soils. Ivanov,[16] for example, in outlining the proximate system of analysis, says that it was verified in his laboratory, and notes its simplicity and convenience. He suggests combination of the cold and hot water extracts into a single hot water extract; combination of the ether and alcohol extracts; and determination of the nitrogen and ash in the various fractions.

OTHER METHODS OF PROXIMATE ANALYSIS

A number of other methods of proximate analysis of plant materials and their decomposition products have been proposed. These methods can be divided into two groups, as follows: 1. Those which make use of different chemical procedures in carrying out the specific analyses, or which go into further detail in analyzing certain groups of constituents, or which take into consideration other chemical complexes. Here belong the methods of Phillips, et al.,[37] Bertrand,[3] and Norman.[32] 2. Those methods which take into consideration the "humic substances" produced as a result of the decomposition of plant materials in soils or in composts. These range from the strictly "humic acid" procedures, based on alkali extractions, to methods of proximate analysis which also give consideration to the "humic acid" fractions.

Among the latter, it is sufficient to mention the methods of Pichard,[38] Groszkopf,[13] Oden and Lindberg,[34] Thiessen and Johnson,[65] and Dragunov.[9]

In Pichard's method, the material was treated first with ether, benzol-alcohol, hot water or, in the case of manure and soil, with cold 2 per cent HCl. It is repeatedly extracted with 2 per cent NH_4OH at 60° and treated with hot 2 per cent HCl for hemicelluloses, with phenol at 180° C. for lignins, and with the Schweizer reagent for cellulose. The residual material is designated as the "humin" fraction. This method is fairly complete. It suffers, however, from being too complicated, from the unnecessary attempt to distinguish between "humic acid" (NH_4OH-soluble fraction) and the "humin" fractions, and especially from failure to recognize the nitrogenous constituents as a separate fraction.

Groszkopf[13] determined the ether, alcohol, and water-soluble fractions; cellulose by the Cross and Bevan method; the mixed lignin and "humic bodies" by the acid method, the latter by treatment with acetyl bromide; "humic acid" by ammonia extraction; and the pentosans by the furfuraldehyde method. This system is open to considerable criticism since no attention was paid to the hemicelluloses as a whole or to the nitrogenous bodies, and since the cellulose was determined by a method which included other substances in addition to true cellulose. Furthermore, considerable confusion may arise in the recognition of "humic acids" and "humic bodies" as two individual entities. Still more open to criticism, because of inaccuracy and unnecessary complications, is the method of Oden and Lindberg and its modification by Thiessen and Johnson.

The method of Dragunov is a modification of the proximate system of analysis. The material left after the hot water extraction is divided into two portions, one of which is treated with acids as in the original method, and the other is treated with hot 2 per cent NaOH and the extract precipitated with 10 per cent HCl, thus giving a "humic acid" fraction. The residue is treated with concentrated acid to give the lignin fraction.

The more strictly "humic acid" methods date back to the time when the soil organic matter was considered as merely a mixture of a few simple organic acids. These methods have been variously modified, without apparent success, however, since the "humic fractions" were found to contain a great variety of well-defined chemical constituents. Among the more recent procedures, based primarily upon the recognition of the "humic bodies," it is sufficient to mention the method of Springer,[54] based upon the separation of the "humic bodies" by treatment with acetyl-bromide and with alkalies, by the degree of oxidation, by color, and by chlorination. With the possible exception of the acetyl-bromide fraction, the other groups can hardly be considered as quantitative measurements of the organic constituents of plant substances and their transformation products.

APPLICATION OF THE PROXIMATE SYSTEM TO THE STUDY OF PLANT MATERIALS AND THEIR DECOMPOSITION

One of the early attempts to analyze quantitatively plant materials in the course of their decomposition by microorganisms was made in 1890 by Hébert.[14] He was able to show that in the course of decomposition of straw there was rapid transformation of ammonia into organic nitrogen. This was accompanied by the disappearance of the cellulose and pentosans and by only a limited destruction of the lignin.

Waksman and Tenney[96] have shown that, by the use of the proximate system of analysis, one can measure the rapidity of decomposition of plant materials and indicate the rate of liberation of the nutrient elements, especially the nitrogen, in available forms. They have further shown that the course of decomposition of different plant materials can thus be easily followed and a study can be made of the influence of environment upon the nature of the decomposition processes. On comparing the proximate composition of straw and alfalfa,[61, 62] they found that the latter was high in water-soluble substances, including nitrogen forms, and lower in cellulose and in lignin. The more rapid decomposition of the alfalfa, as compared with that of the straw, and the greater liberation of the nitrogen by the alfalfa could thus be correlated with the respective chemical composition of the two materials.

By the use of a modified system of proximate analysis, in which greater consideration was given to the complexes containing uronic acid and methoxyl, Phillips, et al.[37] also found that, among the major constituents of alfalfa, lignin was most resistant to decomposition, although it undergoes considerable loss under both aerobic and anaerobic conditions; cellulose was decomposed more rapidly, and pentosans even more so; the alcohol-benzene soluble and the water-soluble constituents, as well as the uronic acid anhydrides and proteins, were most rapidly decomposed. Martin[28] has obtained similar results by the use of the regular proximate method.

Bonnet[6] and Babé and Bonazzi[2] have shown that the reduction of cellulose and hemicelluloses accounts for most of the decomposed sugar cane trash, the lignin and ash fractions remaining constant and the nitrogen accumulating as protein. Tamm and Magistad[60] found that the various chemical constituents of pineapple trash increase in the order of resistance as follows: water-soluble fraction, ether-soluble fraction, alcohol-soluble fraction, hemicelluloses, cellulose, and lignin; the crude protein increases as decomposition advances, whereas water-soluble nitrogen decreases. A detailed study of the influence of chemical composition of plant materials upon their decomposition has also been made by Oberholzer.[33] Vartiovaara[71] used the method of proximate analysis to advantage in studying the decomposition of plant mate-

rials and of purified cellulose by various cultures of cellulose-decomposing soil fungi.[75] The method was also used for the analysis of algae and weeds developing in paddy fields and for the study of the decomposition of these plants.[50]

APPLICATION OF THE PROXIMATE METHOD OF ANALYSIS TO THE STUDY OF STABLE MANURES AND COMPOSTS

In the decomposition of stable manures in composts, for the purpose of conserving the nitrogen in manure, for the reduction of its bulk, or for the growth of cultivated mushrooms, it may become necessary to follow the course of decomposition and to determine the changes in the composition of the various constituents in the manure. In order to accomplish this, no other method so far known could be used more conveniently than the proximate system of analysis. The use of the colorimetric method was found[56] to give insufficient results for this purpose; if it is used at all, it must be accompanied by more quantitative methods, such as the proximate method. If decomposition is normal, it will be found that the disappearance of cellulose and hemicelluloses is accompanied by an increase in proteins and in lignins. The rate of decomposition of the lignin will be considerably delayed under anaerobic conditions. This fact was brought out by the early French workers[8] and in recent studies on the composting of stable manure.[79, 81] The proximate method of analysis has found application in comparative studies of different methods of preserving and composting stable manures.[45]

In the study of the preparation of artificial manures or composts of plant residues,[82, 97] the proximate methods yield results which make possible a detailed comparison between these composts and those of stable manures. In the preparation of composts for the growth of cultivated mushrooms, it becomes essential to determine what chemical constituents are used for the nutrition of the mushroom. This determination is made possible by the proximate method of analysis.[79, 84, 85, 87]

APPLICATION OF THE PROXIMATE SYSTEM TO THE STUDY OF GREEN MANURES

By means of the proximate system of analysis, it was possible to establish the existence of a marked difference in the chemical composition of the growing plant with age.[96] In view of the fact that the rate of decomposition of the plant material and the liberation of the nitrogen in an available form are influenced by the chemical composition of the plant, this information enables one to judge the use of different plant materials, at different stages of growth, for green manuring purposes. It was found that when the plant contains 1.7 per cent nitrogen on a dry basis, there is just sufficient nitrogen to bring about active decomposition of the material without the liberation of any nitrogen during the first few weeks of decomposition. These results were

confirmed by Leukel, et al.[26] using a leguminous plant for green manuring purposes. By the use of the proximate method of analysis, Theron and Van Wyk[64] have shown that, in the semiarid and semihumid rainfall areas of South Africa where green manuring proved a failure under conditions of dry land agriculture, there was no difference in chemical nature between the organic matter of the regularly manured and green manured soils and that of soils kept under continuous corn.

Turk[70] analyzed soybean plants at various stages of growth and found, with advancing maturity of the plants, a decrease in total nitrogen and in water-soluble constituents; an increase in alkali-soluble material, carbohydrates, and lignin; and a widening of the carbon-nitrogen ratio. These changes were readily correlated with the rate of decomposition of the plant as a whole and of various parts of the plant, as well as with the rate of liberation of the nitrogen in an available form. Scheffer and Karapurkar[44] used the proximate method of analysis in attempting to establish a relationship between the rate of nitrification of the nitrogen in plant materials and their chemical composition. They found that by correlating the C:N ratio of the plant materials with their proximate chemical composition, one can easily foretell the rate of liberation of the nitrogen in the process of decomposition of the materials. The hemicelluloses and cellulose were found to have an injurious effect upon the process, and the lignins, a favorable effect. The method has also been used in various other investigations on the chemical compositions of legumes and other green manuring plants, their decomposition, and their utilization in orchards and other crop systems.[101]

APPLICATION OF THE PROXIMATE SYSTEM TO THE STUDY OF PEATS

The decomposition processes taking place in the formation of peats are distinctly different from those involved in composts and in the decomposition of mature plant residues and green materials in soil, the former being anaerobic in nature and the latter largely aerobic. The processes of decomposition, as influenced by plant material, reaction, and water relations, and the nature of the resulting humus can be easily measured by the proximate method of analysis. Under the anaerobic conditions prevailing in peat bogs decomposition is very slow. Certain of the plant constituents, depending on the type of peat, are particularly resistant. In forest and lowmoor peats, the lignins may accumulate almost quantitatively and the proteins also increase in concentration. In sphagnum or highmoor peats, the polyuronides and fatty substances are also resistant to decomposition, thus giving rise to a vibrous, highly acid, polysaccharide-rich and nitrogen- and ash-poor type of peat. These differences have been brought out in a series of investigations by Waksman and Stevens.[88-92]

By the use of a slightly modified form of the proximate method, Feustel

and Byers[10] were able to show marked chemical differences in the peat types of the United States. They established differences in the past history of the peat profile and emphasized the need for further study of the lignin-humus complex. Kivinen[21] noted distinct chemical differences among different types of peat: the sphagnum peats were found to be poor in protein and high in cellulose, whereas the Cyperaceae- and Amblystegium-peats were rich in protein and poor in cellulose. Through comparison of peats with the plants from which they originated, it was possible to follow the course of decomposition, namely a reduction in cellulose and hemicelluloses and an increase in protein and lignin, thus characterizing the process of humification of peat. A definite correlation was observed between the botanical composition and the degree of decomposition of peat, on the one hand, and the chemical composition of the organic substances on the other.

The proximate method was also found[86] to be of value in studying the decomposition of the organic substances in the peat, under favorable conditions of moisture and aeration; the tendency was for a somewhat greater decomposition of the non-nitrogenous constituents than of the nitrogenous. This method was also utilized for peat analysis in order to determine the efficacy of the peat bogs for balneological purposes.[53] Although the earlier students of peat believed that it was impossible to establish chemical distinctions between different types of peat, the proximate method made this possible.[4, 24, 41, 100] The proximate method has also been used for evaluating the quality of peat and its decomposition in soils and in composts,[30] as well as in the study of the chemical nature of peat colloids.[40] Ogg[34a] came recently to the conclusion that, in spite of certain limitations, the proximate method of analysis has "opened a rational line of investigation" in the study of peat.

APPLICATION OF THE PROXIMATE ANALYSIS TO THE STUDY OF THE ORGANIC MATTER IN FOREST AND HEATH SOILS

It was shown in 1928[98] that the organic matter in forest soils can readily be studied by the proximate method of analysis. A definite correlation was found to exist between the nature of the plant residues, conditions of decomposition, and type of humus produced. The marked chemical differences obtained in the composition of raw humus and mull soils could thus be explained. Melin[29] pointed out the existence of a great dissimilarity between the humus of different forest types, which could easily be measured by the activities of microorganisms. The decomposition of wood under both aerobic and anaerobic conditions was shown to be microbiological in nature; this could be established by measuring the changes in the chemical composition of the various organic constituents and comparing them with the corresponding changes brought about by microorganisms in the decomposition of other plant materials.[92]

Wasowicz,[99] using the proximate method of analysis, came to the conclusion that, in the formation of alpine soils, plant associations exert very little if any influence upon the chemical composition of the organic matter in those soils. Climate was found to be the chief factor, followed by physical soil conditions and nature of subsoil. Swederski[59] was able to show that humus in the alpine meadows of the Carpathian mountains contains large amounts of fatty and waxy substances, hemicelluloses, and lignin-humus complexes. The slow decomposition of this humus was correlated with the unfavorable soil conditions for biological activities. The differences in the chemical composition of the humus formations were also correlated with the soil types.

Schmuziger,[47] and Pallmann, Hasler, and Schmuziger[36] analyzed a number of alpine soil profiles and formulated, on the basis of these results, a theory of podzolization. As a result of decomposition of the organic constituents in the A_0 horizon, the A_1 horizon is produced; new products of microbial synthesis, namely, proteins and hemicelluloses, are added to the decomposition products. The A_1 horizon is the "deliverer" of mobile organic substances to the lower horizons. These organic complexes saturate their ionogenic surfaces by adsorbed electrostatic ions; among the organic micrones, the proteins show potentially the greatest degree of dispersion. The high molecular and thickly dispersed lignin (with lower hydrophily and smaller exchange capacity) shows the smallest division tendency. The micelles enriched with H^+, Na^+ and K^+ move as stable highly dispersed phases from the A_1 toward the deeper mineral A_2 horizon, the proteins, partly combined as ligno-proteins, showing the greatest mobility; the less mobile lignin and the remaining organic constituents remain largely in the residue. The mobile organic bodies find an opportunity, in the other mineral horizons, to combine with inorganic substances and give rise to "humus-sesquioxides" which go through the acid, electrolyte-poor A_2 horizon and enrich the little-weathered petrogenic soil layer. In a subsoil containing calcium, the mobile phases are coagulated, either isoelectrically or electrolytically, into the B horizon. This system of podzolization is far more logical and defined better chemically than are those systems based on the "humic acid" and "crenic acid" hypotheses.

Recently Gaarder and Alvsaker[11] made a detailed study of the chemistry of humus in virgin soils of West Norway. They were able to establish differences in the chemical composition of humus, not only for different soil types but also within the same type, as influenced by the nature of the subsoil. The humus as a whole was characterized by a high carbohydrate and a low protein content, abundant rainfall being responsible for the removal of the nitrogenous constituents. The humus was found to be incompletely decomposed and to contain insufficient nitrogen for microorganisms and for plant growth. Decomposition of the carbohydrates was accompanied by an increase in protein and in lignin content. The lack of sufficient bases in the mineral part

of the soil was responsible for a strongly acid reaction and for the weakly decomposed humus, which, as a result of a high rainfall, loses much of its protein and ligno-protein and becomes less suited to normal decomposition and good growth of plants.

Shewan has shown[48, 49] that, by the use of the proximate method, forest soils can be divided into two groups, the mull type and the raw humus type. In the first, hemicelluloses persist in the lower profile layers, and the lignins tend to decrease; in the second, the hemicelluloses disappear, and the lignins increase. This division corresponds to the ecological system of foresters and the biological system of Falck, who distinguished between "corrosion," or the decomposition of both cellulose and lignin, and "destruction," or the decomposition of cellulose and the accumulation of lignin. The relation between forest cover and the chemical nature of resulting humus has been further investigated by Remezov.[43a]

APPLICATION OF THE PROXIMATE ANALYSIS TO THE STUDY OF ORGANIC MATTER IN MINERAL SOILS

Remezov[42] and other Russian investigators[22, 23, 67] made extensive use of the method of proximate analysis for characterizing the organic matter in various soil types of the U. S. S. R. They found that in the humus of serozems, proteins predominate over the lignins, while the cellulose and hemicelluloses are lacking. In chestnut soils, hemicelluloses are present, and lignins abound over proteins. In chernozems, lignins are most abundant, exceeding proteins by 2-2.5 times. In podzols, the organic matter consists about equally of cellulose, lignins, and proteins. Waksman[72, 77, 83] pointed out a change in composition in the organic matter of the soil as one proceeds from the podzols to the serozems. Smolik[52] also used the method for distinguishing the chemical nature of humus in the climatogenetic soil types. Podzols were found to be characterized by a high lignin, hemicellulose, and cellulose content and by a low protein content as compared with chernozems; the water-soluble part of the humus in podzols was higher than in brown soils and black soils. An earlier modification of the proximate method was used also in the study of the organic matter in Japanese soils.[35]

Theron and Van Niekerk[63] found that the humus of the black turf soils in South Africa is resistant to decomposition, because it contains a high proportion, about 60 per cent, of the lignin-protein complex, whereas in the adjoining light colored soils, this fraction makes up only 45 per cent of the organic matter. The chemical composition of this complex was also found to be different: that of the lighter soils had a higher content of nitrogen. By the use of a modification of the proximate method, Kamerman and Klintworth,[17] in a study on the influence of fertilizers upon the nature of the soil organic matter, found that although the total nitrogen is not markedly af-

fected by the addition of nitrogen fertilizer, the hydrolytic nitrogen shows a substantial increase; the same was true, even to a more marked extent for the relation between total carbon and hydrolyzable carbon. The ratio of the latter to hydrolyzable nitrogen gives a fair indication of the effect of nitrogen fertilizer upon the soil. Blok[5] found that fertilization of soil and the nature of the crop grown had an influence upon the chemical composition of the soil humus, especially upon the fatty and waxy substances and the carbohydrates.[19, 60a]

By making use of the proximate system of analysis, McGeorge[27] was able to establish the relation between the various chemical constituents of the soil organic matter and the base exchange capacity of soils; lignins and lignin-like bodies were found to be largely responsible for the replacement capacity.

APPLICATION OF THE PROXIMATE ANALYSIS TO THE STUDY OF THE ORGANIC MATTER IN SEA AND LAKE BOTTOMS AND TO THE ORIGIN OF PETROLEUM

A study of the proximate composition of marine humus, or the organic matter in sea bottoms, revealed the fact[77] that this humus consists of lignins and their derivatives; proteins; carbohydrates, principally polysaccharide hemicelluloses and polyuronides; and small amounts of fatty and waxy substances. On the basis of this composition, the theoretical carbon content of the humus was calculated as 52.8 per cent, and a factor 1.887 was suggested for determining the humus content on the basis of the organic carbon. It was further suggested that a detailed study of the marine humus may throw light upon its origin. Kusnetzow[25] and Speranskaja[53a] were able to correlate the rate of decomposition of the organic matter in lake bottoms and the rate of gas evolution with the nature of the organic matter, as determined by the proximate method.

Trask[68, 69] has shown that the organic constituents of recent marine sediments consist largely of lignin-humus complexes and of nitrogenous bodies, whereas pigments, waxes, and carbohydrates are present only in small quantities. On the basis of these data, he suggested that the changes that occur during and after diagenesis are a partial destruction of the nitrogenous materials, a disappearance of the carbohydrates, a relative increase in resistant complexes, and a generation of bituminous and petroleumlike substances.

APPLICATION OF THE PROXIMATE ANALYSIS TO THE STUDY OF THE ORGANIC MATTER IN SEWAGE AND IN GARBAGE

The proximate system of analysis was found[15] to be applicable to the study of fresh solids and ripe sludge in sewage. By this method, one could trace readily the process of decomposition of organic matter in sewage. Fats were

found to decompose rapidly under anaerobic conditions; the hot alcohol soluble constituents were resistant to decomposition; cellulose was more or less completely decomposed; hemicelluloses tended to persist, a fact which may indicate their resynthesis by microorganisms; lignins accumulated; soluble forms of nitrogen decreased, and insoluble forms increased. Similar applications of the method could be made to the study of garbage decomposition. It is of interest to note that microbial cell substance can also be analyzed by means of the proximate method. It was shown,[39] for example, that the composition of the mycelium of *Aspergillus niger* can be modified by the addition of a trace of zinc to the medium. The chemical composition of the edible mushroom, *Agaricus campestris*,[84] pointed to an interesting correlation between composition of substrate and of organism grown upon it.

APPLICATION OF THE PROXIMATE SYSTEM OF ANALYSIS TO THE EVALUATION OF COMMERCIAL HUMUS PREPARATIONS

In recent years, many organic matter preparations have been placed on the market as organic fertilizers, under various trade names. They are usually prepared from peat, garbage, sewage residues, and composts of plants and animal (fish) residues. Some are enriched with lime, ammonia, or a mixture of inorganic fertilizers. In order to determine the origin of these materials, so as to throw light upon their fertilizing value, it is not merely sufficient to analyze for total nitrogen and mineral constituents, since their major claims are based upon the organic matter, its origin, rate of decomposition, and possible effects upon soils and crops. The proximate system of analysis may prove a valuable aid in this connection.[55] A high cellulose, high hemicellulose, and high fat content indicates origin from highmoor peat; a high lignin, a high but resistant nitrogen, and a low fat and wax content indicates origin from lowmoor peat; readily hydrolyzable nitrogen compounds and a certain amount of cellulose and hemicellulose indicate compost origin.

SUMMARY

The proximate system of analysis of plant materials and their decomposition products offers a valuable aid in determining the nature of the material, the chemical changes involved in its decomposition, the nature of the humus produced, and its resistance to further decomposition. In spite of the limitations, in failing to define definitely some of the humus fractions and to account for certain highly specific plant constituents, this method proves valuable in throwing considerable light not only upon the origin of humus but upon its chemical nature as well.

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MICROBIAL ACTIVITY IN RELATION TO ORGANIC MATTER TRANSFORMATION IN THE SOIL

S. C. VANDECAVEYE

State College of Washington, Pullman, Washington, U. S. A.

THE transformation and mineralization of organic matter in the soil is primarily a function of the soil microflora. The humus and its decomposition products which result from microbial activity contribute much to the inherent soil characteristics developed in the process of soil formation and to that property known as soil fertility. The nature of the humus is determined in large measure by the kind and activity of the microflora producing the humus, the chemical composition of the organic materials from which it is derived, and the prevailing climatic conditions under which it is formed. Since the soil microflora is profoundly influenced by the chemical composition of the organic matter constituting the main food supply as well as by the climatic conditions and the inherent soil properties under which its activities are carried on, the study of its diverse functions in relation to organic matter transformations, though intriguing, is complicated by many interrelated factors. The purpose of this paper is to present briefly certain studies made by the writer and his collaborators in an attempt to elucidate some of these factors.

EXPERIMENTAL PROCEDURES

The principal studies under consideration consisted of periodical determinations of reaction; CO_2 production; and numbers of bacteria, fungi, and actinomyces in soils under laboratory and field conditions. In certain cases these determinations were supplemented by chemical analyses of the organic residues added to certain soils and of the organic matter in the soils in an attempt to correlate microbial behavior with the nature of the organic matter serving as the main source of food. The soils used represent various genetic soil groups.

The laboratory studies were made on thoroughly mixed untreated samples of 1500 to 2000 gm. taken from the A and B horizons of virgin soils, and also on similar samples taken from the surface 8 to 10 inches of cultivated and virgin soils, which were treated with 1 or 2 per cent of various finely ground organic residues reinforced with sufficient quantities of soluble

nitrogen to make their total nitrogen content equivalent to 3 per cent. This supplemental nitrogen was added to insure the microbial development against any possible shortage of available nitrogen. The moisture content of the soils in the laboratory was adjusted to the normal field moisture percentage, which was maintained within narrow limits by additions of water when necessary during the incubation period. The incubation temperature ranged between 22 and 28°C., depending upon the nature of the experiment.

The soils studied in the field were sampled at various depths in the surface 10-inch layer over a period of 15 months, at monthly intervals whenever possible. The samples were taken to the laboratory where they were thoroughly mixed, air dried quickly, and then prepared for microbial counts.

The CO_2 determinations were made by the aspiration method; and the microbial counts, by the plate culture method using selective culture media. The details of the methods are described elsewhere.[6] To insure thorough mixing the soils were passed twice through a 6-mesh sieve before samples were removed for plating, and the average values of triplicate plates were recorded.

The chemical analyses of the organic residues and soil organic matter were made by the method of proximate chemical analysis of Waksman and Stevens[11] modified to the extent that the carbon in the extracts and residues was determined directly by the wet combustion method of Friedemann and Kendall,[3] and the fractionations were limited to the water-soluble, the 2 per cent hydrochloric-acid-soluble, the 70 per cent sulfuric-acid-soluble, the sulfuric-acid-insoluble, and the total carbon and nitrogen. The Kjeldahl method[1] was used for the nitrogen determinations, and the quinhydrone and glass electrode methods were used for the pH determinations.

EFFECT OF PLANT RESIDUES ON THE MICROBIAL ACTIVITY IN DIFFERENT SOIL TYPES

The well-known fact that the activity of the soil microflora is stimulated by additions of plant residues to the soil is vividly demonstrated by profuse CO_2 evolution and rapid multiplication in numbers of bacteria, fungi, and actinomyces immediately following the incorporation of these materials into the soil. Since the evolution of CO_2 is a product of microbial activity it has been used often as a measure of the intensity of this activity and, indirectly, of the numerical development of soil microbes. Our experiments have shown invariably that under optimum moisture conditions at laboratory temperatures ranging between 20 and 26° C. the maximum production of CO_2 in soils which had received 1 or 2 per cent plant residues occurs within the first three to five days following the incorporation of these plant materials into the soils, whereas maximum microbial numbers do not occur until 10

or 15 days later. Obviously the lag between these maxima is too great to use CO_2 production as an accurate measure of microbial development with respect to numbers, although it may possibly serve as an approximate index of the total microbial activity.

TABLE 1

MICROBIAL NUMBERS AND CARBON AND NITROGEN CONTENTS OF VARIOUSLY TREATED PALOUSE AND MELBOURNE SILT LOAMS

Soil and treatment	Averages of 5 counts during period of maximum activity of 40 days			C as CO_2	Organic C	N	C-N ratio	pH value
	Bacteria	Fungi	Actinomyces					
	thousands/gm.	*thousands/gm.*	*thousands/gm.*	*gm./kgm.*	*per cent*	*per cent*		
Palouse silt loam, untreated	19,738	32	1,306	1.32	2.569	0.196	13.0	6.7
Palouse silt loam+1 per cent wheat straw	25,800	242	3,174	3.31				
Palouse silt loam+1 per cent sweet clover hay	32,940	156	4,780	3.30				
Melbourne silt loam, untreated	3,180	346	179	0.80	4.674	0.249	18.8	5.3
Melbourne silt loam+1 per cent wheat straw	2,012	4,680	149	2.18				
Melbourne silt loam+1 per cent sweet clover hay	5,210	4,840	309	3.02				

The influence of the chemical composition and of the quantity of available organic food and the effect of certain chemical and physical properties of the soil on the activity of the soil microflora have been subjects of study by many investigators, whose works have been reviewed comprehensively by Waksman.[8] Because of the simultaneous functioning of various closely interrelated factors, the cause or causes for many of the known differences in microbial activity in different soils are still obscure. The composition of the microflora and the numbers of organisms composing the principal groups of microbes taking part in organic matter transformations are not necessarily correlated with such factors as amount of soil humus, carbon and nitrogen relationships of soil humus, pH values of the soil, and calcium or other nutrient contents of the soil. This was convincingly demonstrated in certain of our experiments in which cultivated and virgin forest and grassland soils of similar texture were used. One of these cultivated forest soils is Melbourne silt loam with an organic matter content of 8.1 per cent, and one of the cultivated grassland soils is Palouse silt loam containing 4.4 per cent organic matter. The virgin forest soil in question is Helmer silt loam with an organic matter content of 4 per cent and a pH value of 6.2, and the virgin grassland soil is Palouse silt loam with an organic matter content of 6 per cent and a pH value of 6.4. The Palouse and Helmer soils are derived from identical parent material and are situated in adjoining areas. Certain

pertinent data regarding the microbial activity in these cultivated and virgin forest and grassland soils are presented in Tables 1 and 2. Although it is realized that averages of numbers of organisms determined even at relatively close intervals may not faithfully reflect a rapidly fluctuating microbial population which is only partially represented by plate counts, the fact that the principal species of the various microbial groups seem to reappear repeatedly in dominant numbers should be highly significant in estimations of the nature of microbial actions.

TABLE 2

AVERAGE NUMBERS OF BACTERIA, FUNGI, AND ACTINOMYCES AT VARIOUS DEPTHS IN VIRGIN PALOUSE AND HELMER SOILS UNDER FIELD CONDITIONS

Averages of 13 periodical determinations during 15 months

	Virgin Helmer silt loam			Virgin Palouse silt loam		
Depth.....*inches*	0–0.5	0.5–4	4–10	0–0.5	0.5–4	4–10
Bacteria.....*thousands/gm.*	3,388	2,018	573	5,950	1,863	1,234
Fungi.....*thousands/gm.*	653	182	35	198	152	41
Actinomyces....*thousands/gm.*	755	541	232	1,350	1,023	402
Carbon.....*per cent*	2.32			3.46		
Nitrogen.....*per cent*	0.100			0.209		
C-N ratio.....	23.2			16.5		
pH value.....	6.2			6.4		

As may be noted from the data in Tables 1 and 2, the cultivated Melbourne forest soil with 8.1 per cent organic matter supported only one sixth as many bacteria and one seventh as many actinomyces as the cultivated Palouse grassland soil with 4.4 per cent organic matter. Although the Melbourne soil contained nearly 11 times as many fungi as the Palouse soil, a factor which might partly counterbalance the differences in bacterial and actinomycete numbers, the total microbial activity as measured by CO_2 evolution was considerably less in the Melbourne soil than in the Palouse soil, indicating that microbial activity in these soils is not proportional to the organic food supply. A similar tendency, though less pronounced and subjected to seasonal fluctuations, as discussed elsewhere,[7] was manifested in the two virgin soils, which on account of their common origin and climatic environment are more closely related in their genetic and chemical characteristics than the two cultivated soils and, hence, would be expected to possess less divergent microflora. Yet an inverse relationship between the organic food supply and the microbial activity exists in both the cultivated and the virgin soils, despite the insignificant difference in reaction between the two virgin soils in contrast to the marked difference in pH values of the two cultivated soils. The latter factor might be of sufficient magnitude to

cause significant differences in the microbial flora and activity, but it does not account for the observed inverse relationships.

The specific adaptability of certain food substances to specific groups of soil microbes is well known. When simple sugars, such as glucose, are supplied to mixed cultures of microbes such as prevail in the soil, the bacteria will easily outgrow the fungi and actinomyces, particularly in the presence of small quantities of nitrogen. The development of fungi is stimulated more than that of the other two groups by additions of celluloses and pentosans to soils well supplied with available nitrogen. The actinomyces, on the other hand, seem to thrive better on lignins than do the other two groups. The degree of stimulation exerted by specific food substances on the development of specific groups of microbes is modified, however, by inherent soil properties, and consequently the addition of these materials to different types of soil may not produce the same results. It may be noted from the data in Table 1 that the addition of 1 per cent sweet clover hay to the Melbourne and Palouse soils produced about the same stimulating effect on bacterial development in the two soils but caused a fourteenfold increase in numbers of fungi in the Melbourne soil and only a fivefold increase in numbers of these organisms in the Palouse soil. The same substance resulted in more than a threefold increase in the actinomycete population in the Palouse soil and less than a twofold increase in the Melbourne soil. The addition of 1 per cent of wheat straw to these soils resulted in a very marked increase in population of all three groups of microbes in the Palouse soil and in the fungal population of the Melbourne soil, but it caused a reduction in numbers of bacteria and actinomyces in the latter soil. As available nitrogen was not a limiting factor in this case, and as temperature and moisture factors were the same for the two soils, the cause of these different effects evidently lies in differences in the nature of the humus and other soil properties. Similar effects were produced by the addition of 2 per cent Helmer soil duff and Palouse soil litter respectively to separate samples of virgin Palouse and Helmer soils in another experiment.[6] The Helmer soil duff stimulated bacterial and fungal development more an actinomycete growth less in the Palouse soil than in the Helmer soil. The Palouse soil litter produced similar effects on the bacterial growth in the two soils but resulted in a much greater development of actinomyces and a markedly smaller development of the fungi in the Palouse soil than in the Helmer soil.

The previously outlined proximate chemical analysis[6] of the humus and the corresponding organic residues of the virgin Palouse and Helmer soils revealed marked differences in the chemical composition of these substances in the two soils. Both the humus and the organic residue of the Palouse soil contain much larger quantities of sulfuric-acid-soluble carbon and nitrogen than do the corresponding substances of the Helmer soil. The sulfuric

acid fraction, which comprises 25 to 30 per cent of the total carbon and 40 to 50 per cent of the total nitrogen, contains, among other carbon compounds, hemicellulose and cellulose, as well as a comparatively large percentage of protein. This fraction appeared to be a more suitable food for actinomyces than for bacteria and fungi, for the stimulated development of the actinomyces was roughly proportional to the quantity of hemicellulose, cellulose, and protein in these soils and in the residues added to the soils. Moreover, the maximum numbers of actinomyces occurred only after the numbers of bacteria and fungi had declined, coincidentally with the disappearance of a large percentage of the more readily available food supply, namely, the water-soluble carbon and nitrogen compounds. This corresponds with the sequential microbial activity in the order of bacteria, fungi, actinomyces, and finally aerobic cellulose-decomposing bacteria observed in several of our experiments and noted also by various other investigators, notably Norman[4] and Waksman and Starkey.[10] All the foregoing observations lend strength to the assumption that the nature of the soil humus as well as the composition of the organic residues added to the soil has a profound influence upon the composition and activities of the soil microflora.

AMOUNT AND NATURE OF HUMUS IN RELATION TO MICROBIAL ACTIVITY

The possible effect of different kinds of humus on the microbial activity in the soil was studied further in one of our recent experiments in which representative samples of the A and B horizons of 15 soils representing various genetic types were employed. The microbial and chemical determinations were made in accordance with the methods outlined in the experimental procedure. Some of the pertinent data obtained are presented in Table 3, in which the various soils are grouped under broad genetic soil types.

A notable feature revealed by the data on microbial counts is the extreme variation in numbers of organisms in the A and B horizons of the 15 soil types. In the A horizons the average numbers of bacteria per gram vary from 273,000 to 8,875,000, the numbers of fungi from 2,000 to 739,000, and the numbers of actinomyces from 5,000 to 3,800,000. Thus, under optimum moisture and temperature conditions the A horizons of the soils with the highest counts are capable of supporting 32 times as many bacteria, 369 times as many fungi, and 760 times as many actinomyces as the A horizons of the soils with the lowest counts. The differences are less pronounced in the B horizons, which indicate that the soils with the highest counts can support 17 times as many bacteria, 259 times as many fungi, and 98 times as many actinomyces as the ones with the lowest counts.

The data on total organic carbon, though showing marked differences in values, disclose that the extreme variations in carbon content of the soils range from 8.9 to 0.75 per cent or a ratio of 11.8 to 1 for the A horizon and

TABLE 3

MICROBIAL NUMBERS, CARBON AND NITROGEN RELATIONSHIPS, AND pH VALUES IN DIFFERENT CLIMATIC SOIL TYPES

Genetic group	Soil type	Soil number and horizon	Depth	Average numbers of 4 monthly counts			Organic C	N	C–N ratio	pH value
				Bacteria	Fungi	Actinomyces				
			inches	*thousands/gm.*	*thousands/gm.*	*thousands/gm.*	*per cent*	*per cent*		
Sierozem soil	Ephrata silt loam	1A	0–10	4,525	33	2,112	0.99	0.063	15.77	7.1
		1B	10–36	2,248	13	195	0.87	0.041	21.58	7.0
	Mohave clay loam	2A	0–15	6,250	2	775	0.75	0.050	15.05	7.9
		2B	40–50	7,525	2	514	0.30	0.027	11.09	7.9
	Ritzville fine sandy loam	3A	0–12	3,700	30	173	2.19	0.148	14.81	6.6
		3B	12–24	5,198	4	648	0.83	0.065	12.63	7.2
Chernozem soil	Barnes clay loam	4A	1–12	5,000	10	2,375	4.56	0.372	12.25	6.9
		$4B_1$	16–28	5,550	1	963	1.39	0.099	14.08	7.9
	Garrison gravelly loam	5A	0– 8	3,700	46	1,725	3.90	0.226	17.25	7.5
		5B	8–18	5,250	9	1,275	1.20	0.109	11.00	7.0
	Palouse silt loam	6A	0–12	2,950	36	1,025	2.80	0.164	17.02	6.4
		6B	18–40	2,950	2	825	1.09	0.047	23.06	6.6
Prairie soil	Houston clay loam*	7A	0–11	8,825	3	1,438	2.01	0.189	10.63	7.4
		7B	11–33	7,650	6	1,125	1.39	0.118	11.70	7.7
	Spanaway gravelly sandy loam†	8A	0–12	898	179	340	8.60	0.693	12.41	5.0
		8B	12–24	1,340	98	133	3.03	0.221	13.75	6.0
	Spanaway gravelly sandy loam‡	9A	0–16	655	171	1,238	8.98	0.391	22.94	5.4
		9B	16–30	1,585	69	195	0.75	0.025	29.80	6.7
Gray-brown podzolic soil	Lynden sandy loam	10A	0– 8	5,825	35	1,000	1.34	0.054	24.80	6.6
		10B	8–28	2,968	18	828	1.29	0.041	31.76	6.6
	Alderwood sandy loam	11A	0– 6	1,413	65	973	2.05	0.079	25.84	5.9
		11B	6–20	453	17	116	1.62	0.058	27.72	5.9
	Helmer silt loam	12A	0–10	4,525	739	3,800	1.73	0.077	22.56	6.2
		12B	15–35	1,015	259	718	0.75	0.032	23.15	5.7
	Rainier sandy loam§	13A	1.5– 5	325	171	165	1.42	0.030	47.01	6.4
		13B	8–18	623	164	36	0.73	0.014	50.69	6.3
Podzol soil	Lakewood sandy loam	$14A_1$	0– 3	273	419	5	1.24	0.027	45.83	5.4
		14B	17–33	835	18	13	0.57	0.009	63.23	6.2
	Greenville fine sandy loam‖	15A	0– 3	4,600	160	2,413	1.73	0.072	24.06	6.3
		$15B_2$	24+	2,208	7	993	0.76	0.023	32.93	5.7

* Rendzina soil; † prairie soil; ‡ forest soil; § immature podzol; ‖ lateritic soil.

from 3.03 to 0.30 per cent or a ratio of 10 to 1 for the B horizon. No consistent correlation is indicated between numbers of microbes and humus content in the A or B horizons of these soils. Although the A and B horizons of the sierozem, chernozem, and prairie soils, in general, contain a much larger bacterial population and a much smaller fungal population than the forest soil (except soil 8 in the prairie soil group which behaves more like a forest soil), considerable variation in numbers of these organisms exists among soils belonging to the same genetic group. This is in accord with the observations of Tunanin[5] for Canadian soils. The actinomyces, though generally more numerous in the chernozem and prairie soils than in the sierozem and forest soils, were influenced less by genetic soil characteristics than were the bacteria and fungi, except in the two podzol soils in which the acid reaction probably is a limiting factor for actinomycete development.

Another significant point, contrary to the contentions in the older soil microbial literature but in conformity with the observations of several investigators including Brown and Benton[2] and Tunanin,[5] is that under proper moisture and temperature conditions the soil in the B horizon is capable of supporting a very active bacterial flora. Four of the pedocals and four of the pedalfers, or the majority of the soils listed in Table 3, supported a larger bacterial population in the B horizon than in the A horizon despite the relatively low humus content of the B horizons. The fungal and actinomycete populations were smaller in the B horizons than in the A horizons in most cases. None of the foregoing differences in microbial activities can be explained, however, by the differences in humus content, and the contention already made that no consistent correlation exists between microbial numbers and humus content in these soils is further supported.

In consistency with the marked variation in organic carbon content of the soils under consideration, the results of the fractionation of the humus disclose differences of similar or even greater magnitude in the carbon and nitrogen contents of the hydrochloric-acid-soluble, sulfuric-acid-soluble, and sulfuric-acid-insoluble fractions. The data, which will be reported in a subsequent publication, show, for instance, that whereas the high and low values for the total organic carbon in the A horizons are in the ratio of 12 to 1, the corresponding values for the carbon in the hydrochloric-acid-soluble, sulfuric-acid-soluble, and sulfuric-acid-insoluble fractions are in the ratios of 20 to 1, 13 to 1, and 12 to 1 respectively. Similar differences occur in the nitrogen content of these fractions. No correlation is evident between marked differences in amounts of either carbon or nitrogen in the various fractions of the humus in either the A or B horizons of these soils and the highly significant differences in microbial activity. Since varying quantities of specific food substances in the proper proportion may not influence microbial growth so much as do unbalanced proportions of these substances,

the carbon-nitrogen relationships and the percentages of water-soluble, hydrochloric-acid-soluble, and sulfuric-acid-soluble carbon and nitrogen in the humus should not be overlooked.

The carbon-nitrogen ratios of the humus in the A and B horizons of the sierozem, chernozem, and prairie soils are distinctly lower than those of the humus in the gray-brown podzolic and podzol soils. This is in agreement with the results of Waksman and Hutchings[9] and also corresponds broadly with the larger numbers of bacteria and the smaller numbers of fungi in the former than in the latter soils; but it does not account for the generally large differences in numbers of actinomyces between the A and B horizons and among the various genetic soil types, or for the small numbers of bacteria and the large numbers of fungi in the Spanaway prairie soil which contains a large amount of humus with a low carbon-nitrogen ratio.

In contrast to the large differences in amounts of carbon and nitrogen in the various organic fractions in these soils, the percentages of both are much less variable. The percentages of water-soluble carbon and nitrogen in the humus are uniformly small. The values for hydrochloric-acid-soluble, sulfuric-acid-soluble, and sulfuric-acid-insoluble carbon and nitrogen in percentages of the total carbon and nitrogen fluctuate to some extent, but the differences among the genetic soil groups are not outstanding, except that the sums of the values for hydrochloric-acid-soluble and sulfuric-acid-soluble carbon and nitrogen are generally larger for the soils in the gray-brown podzolic and podzol groups than those for the other genetic groups. These differences in values correspond with the results of Waksman and Hutchings,[9] but the higher values for sulfuric-acid-insoluble carbon reported by them for the chernozem soils in comparison to those for the podzols were not uniformly realized in the soils used in our investigations.

The relatively small differences in percentages of hydrochloric-acid-soluble and sulfuric-acid-soluble carbon and nitrogen in terms of the total carbon and nitrogen content of the humus of the 15 soil types are insufficient to account for the significant differences in numbers of actinomyces in these soils. A comparison of this condition with the apparent stimulating effect exerted on actinomycete development by the much larger differences in amounts of hydrochloric-acid-soluble and sulfuric-acid-soluble carbon and nitrogen between the Palouse soil and its homologous organic residue and the Helmer soil and its homologous organic residue, discussed earlier, complicates these relationships. The first condition receives added support, however, in that the relatively small differences in percentages of carbon and nitrogen in any of the other organic fractions of these various soil types are not of sufficient magnitude to serve as an explanation for the observed large and significant differences in numbers of organisms in the three major microbial groups. Apparently the proximate analysis of the humified organic

matter in the soil is inadequate, at least in its present state of development, to serve as a single tool in detecting the cause of these important differences in microbial activity. Perhaps the picture of the activity of the micropopulation in soil organic matter transformations is complicated also by other factors such as the supply of available mineral nutrients and the physical and chemical conditions of the soil, which should receive more consideration as a means of supplementing the method of proximate analysis of the humus. After all, the study of the microbiological phase of the soil is still in its infancy, but the importance of the role played by the humus and the activity of the micropopulation in organic matter transformation, in the development of inherent soil properties, and in soil fertility has become increasingly evident from the many valuable contributions made in recent years. Thus this field of investigation is immensely rich in possibilities for further scientific discoveries and for the practical applications of such discoveries in agriculture.

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THE OCCURRENCE IN SOIL OF BACTERIA, ACTINOMYCETES, AND MOLDS CAPABLE OF TRANSFORMING TYROSINE TO MELANIN OR OTHER PIGMENTS

CHARLES E. SKINNER

University of Minnesota, Minneapolis, Minn., U. S. A.[1]

SEVERAL bacteria, actinomycetes, and molds are known to produce black or black-brown pigments on certain culture media. The pigment or pigments, usually termed "melanin," have in most cases not been sufficiently identified by chemical or physicochemical means, and that they are true dopa melanin has been proved in only rare instances.

An examination of many soils has revealed a very large population of these organisms, not only of actinomycetes, which was expected, but of Eubacteriales and Eumycetes as well. In most soils, many more than 100,000, often more than 1,000,000, true bacteria and several thousand molds as well as the usual number, in the hundreds of thousands, of actinomycetes, all producing these pigments, were encountered.

The soils were plated quantitatively on Conn's sodium asparaginate agar with glucose in place of glycerol and with the addition of 0.1 per cent tyrosine, and in 1 per cent peptone agar with and without 0.1 per cent glucose. Tryptone peptone gave better results than other brands. About 20 cc. of medium was used per petri plate. As fast as colonies developed around which a black, brown, or pink color developed, they were isolated.

Although bacteria of different morphological types were isolated, most of them were of two types, probably two species. One, identified as *Bacillus niger*, was a Gram-positive rod which formed spores centrally or subterminally with a swelling of the cell. The pigment which developed was black or brown turning black. No intermediate red color was ever seen on any medium. The pigment developed on 1 per cent peptone agar (Tryptone) and on Conn's agar with glucose substituted for glycerol when 1 per cent peptone, 1 per cent casein, or 0.1 per cent tyrosine was added, but not when 0.1 per cent histidine or 0.1 per cent glycine was added. In some cases a slight pigment developed belatedly with the addition of 0.1 per cent tryptophane. The other type of bacteria, which was encountered more often,

[1] Department of Bacteriology and Immunology. This investigation was aided by a grant from the Graduate School.

was a very large Gram-positive rod with spores. This species formed a red or pink pigment which on long incubation turned brown and finally, in basic media, brown-black. The red pigment changed at once to black when it was extracted with weak NaOH.

The molds which formed these pigments varied in morphology. Many of them have been identified as belonging to well-known genera of Mucedinaceae or Mucoraceae. It was difficult to determine with certainty whether the pigment of the Dematiaceae was entirely in the mycelium or had diffused. These were not studied extensively. Most of the molds produced either the brownish black pigment at once, or else the red pigment which turned dark brown very slowly over several weeks. A few strains passed through the red to brown to black transformations within a week or two. As with bacteria, the pigments were formed only on media containing free or combined tyrosine, although in a few cases a slight color developed belatedly on media containing tryptophane.

Many actinomycetes, like true bacteria and fungi, were found to produce the black pigment at once, without an intermediate red stage, only on media containing tyrosine; others produced a bright red pigment which changed very slowly over several weeks to brown. Some of these latter strains also produced the red color from phenylalanine. Many species of actinomycetes obviously are capable of forming these pigments from tyrosine. The attempt to lump together as one species all the soil "chromogenus" strains or to label any one of the plant pathogens, as *"Actinomyces chromogenus,"* is neither good taxonomy nor is it in the interest of scientific clear thinking. A more thorough study of the tyrosinase reaction of the actinomycetes has already been published.

The identity of the pigments is being studied by Dr. E. Arnow by spectroscopic methods. The black pigment is soluble in weak alkalies, is precipitated by hydrochloric or other acids, and is insoluble in alcohol and ether. It disperses in neutral water and alcohol. It will be noted that there is a strong resemblance between this pigment and Oden's "humus acid" or "the humic acid portion of humic material" of Page. The red pigment is promptly changed to black when the medium in which it has developed is heated with weak NaOH, and the character pigment thus formed is like that of the black pigment just described. From our knowledge of the chemistry of melanin formation, it is likely that the red pigment is the precursor of the black, and enzymes for this transformation are produced by one of these species and not by the other.

Since the soil has been shown to harbor such a large population of bacteria, molds, and actinomycetes capable of transforming free or combined tyrosine into black pigments which have many of the properties of an im-

portant fraction of the soil "humus," and since it is well established that many soil fungi can transform inorganic nitrogen into free and combined tyrosine, the possibility is suggested that part of the soil humus, and incidentally its black color, may be derived from added or synthesized proteins by oxidation by microorganisms.

DECOMPOSITION OF THE ORGANIC COMPOUNDS IN BARNYARD MANURE

N. Bengtsson and Chr. Barthel

Institute of Microbiology, The Agricultural College of Sweden, Ultuna, Sweden

In our earlier investigations of nitrification of stable manure nitrogen in soil, the transformation of only the manure nitrogen was studied. These

TABLE 1

Initial Values* of the Various Samples

Results in mgm. per 100 gm. of sample

Samples	pH	Total N	Ammonia N	Nitrate N
I	8.1	45.1	7.9	0.0
II	8.1	54.4	7.9	9.2
III	8.1	63.6	7.9	18.4
IV	6.1	44.4	7.9	0.0
V	6.1	53.7	17.1	0.0
VI	6.1	62.9	26.3	0.0
VII	6.0	53.7	7.9	9.2
VIII	5.7	62.9	7.9	18.4

* The total N of the sand, 1.3 mgm. for samples I–III and 0.6 mgm. for samples IV–VIII, is included in the values for total N. For all samples, the value of protein N, assumed to be equal to the difference between total N and ammonia N values for the manure, was 35.9 mgm.; the value of α-humus, 110 mgm.; cellulose, 494 mgm.; pentosans, 354 mgm.; lignin (expressed as lignothioglycolic acid), 330 mgm.; and organic carbon, 783 mgm.

investigations have shown very definitely that the protein nitrogen in the manure is mineralized rather slowly. In the experiments here reported the decomposition of the cellulose, pentosans, and lignin in the manure was also studied. Simultaneously (1, 2, 3, and 4 years after the start of the experiments) the α-humus and the organic carbon were determined. The analytical methods used are described in the *Annals of the Agricultural College of Sweden*, vol. 3, pp. 1, 169 (1936).

The manure used was obtained from cows which had been fed on hay, straw, and concentrates. It was 3 months old and contained urine, straw, and chaff. The experiments were made in sand at initial pH values of 6

and 8. In the former case the sand had been washed with hydrochloric acid and water; in the latter, only with water. All the samples contained 7560 gm. dry sand, 840 gm. ground manure, and 4.2 gm. KH_2PO_4. They were

TABLE 2

RESULTS AFTER 1 YEAR

Samples	pH	Total N	Ammonia N	Nitrate N	α-Humus	Cellulose	Pentosans	Lignin	Organic C	Protein N
						Per cent decomposed				
		mgm.	*mgm.*	*mgm.*	*mgm.*					
I...	7.5	40.4	0.1	9.0	89	94	81	38	53	16
II...	7.7	46.1	0.0	13.1	86	94	84	32	55	12
III...	7.6	56.7	0.1	23.5	92	95	83	36	55	12
IV...	5.7	42.4	3.2	5.8	105	77	63	26	41	9
V...	5.6	52.4	11.4	5.8	102	73	61	17	36	4
VI...	5.5	59.9	19.3	5.7	107	75	59	14	36	5
VII...	5.5	47.9	2.1	13.5	102	75	61	17	40	12
VIII...	5.5	56.0	0.9	20.6	102	79	61	13	28	6

inoculated with 120 ml. soil extract which was prepared from two clays with ordinary humus content and with pH values of 5.0 and 7.5 respectively. In addition, the samples received the following treatments:

Sample	I.	No addition
"	II.	6.06 gm. KNO_3
"	III.	12.12 gm. KNO_3
"	IV.	252 ml. *N* H_2SO_4
"	V.	3.97 gm. $(NH_4)_2SO_4$ plus 252 ml. *N* H_2SO_4
"	VI.	7.94 gm. $(NH_4)_2SO_4$ plus 252 ml. *N* H_2SO_4
"	VII.	6.06 gm. KNO_3 plus 252 ml. *N* H_2SO_4
"	VIII.	12.12 gm. KNO_3 plus 252 ml. *N* H_2SO_4

The amount of sulfuric acid added to samples IV-VIII adjusted the acidity to a pH value of 6, as shown by separate experiments. The additions of potassium nitrate and ammonium sulfate, which corresponded to approximately one or two equivalents of the ammonia nitrogen in the manure, were made in order to determine whether the decomposition of the proteins could be accelerated. The water content was adjusted to 15 per cent in all the samples. After thorough mixing, each sample was divided into 21 parts, which were put into 300-ml. glass jars. The jars were stoppered in the manner described in the *Annals of the Agricultural College of Sweden*, vol. 7, p. 123 (1939). After being weighed, the jars were incubated at 20-22°C. When necessary, water was added to replace losses by evaporation.

The initial values are presented in table 1; and the results of the subsequent

determinations, in tables 2-5. In the last four tables the values for total nitrogen, ammonia nitrogen, nitrate nitrogen, and α-humus are reported in

TABLE 3

RESULTS AFTER 2 YEARS

Samples	pH	Total N	Ammonia N	Nitrate N	α-Humus	Cellulose	Pentosans	Lignin	Organic C	Protein N
						Per cent decomposed				
		mgm.	*mgm.*	*mgm.*	*mgm.*					
I...	7.4	40.1	0.2	13.1	80	95	87	43	58	29
II...	7.6	44.8	0.2	17.9	83	97	88	45	59	30
III...	7.5	54.2	0.1	26.8	78	97	88	46	58	28
IV...	5.6	41.1	3.3	8.8	98	77	67	23	37	21
V...	5.6	50.5	10.8	7.9	86	75	65	20	37	13
VI...	5.5	60.3	19.9	8.3	101	74	65	24	37	13
VII...	5.5	49.2	1.5	15.8	98	76	66	24	37	13
VIII...	5.6	55.4	1.0	23.5	97	75	63	20	31	16

milligrams per 100 gm. On the basis of these nitrogen figures and the corresponding initial values, the figures for protein nitrogen decomposed were calculated. The results of the investigation show the following:

TABLE 4

RESULTS AFTER 3 YEARS

Samples	pH	Total N	Ammonia N	Nitrate N	α-Humus	Cellulose	Pentosans	Lignin	Organic C	Protein N
						Per cent decomposed				
		mgm.	*mgm.*	*mgm.*	*mgm.*					
I...	7.1	39.9	0.1	17.3	107	97	90	52	67	41
II...	7.3	44.6	0.1	22.0	93	98	91	54	67	41
III...	7.2	53.5	0.1	31.9	89	98	93	56	69	44
IV...	5.3	43.7	2.4	12.3	116	84	69	23	40	21
V...	5.4	52.4	11.7	10.5	106	81	70	21	39	18
VI...	5.2	60.4	18.7	11.5	120	78	68	11	36	18
VII...	5.3	47.3	0.5	19.1	114	78	69	23	41	25
VIII...	5.4	56.0	1.2	25.7	114	79	69	22	42	21

All the processes of decomposition here studied have proceeded decidedly more rapidly at the alkaline reaction than at the acid one. The additions of nitrogen, by which the ratio of carbon to assimilable nitrogen was narrowed

TABLE 5

RESULTS AFTER 4 YEARS

Samples	pH	Total N	Ammonia N	Nitrate N	α-Humus	Cellulose	Pentosans	Lignin	Organic C	Protein N
						Per cent decomposed				
		mgm.	*mgm.*	*mgm.*	*mgm.*					
I...	6.9	40.9	0.0	19.5	79	99	91	60	70	44
II...	7.1	44.3	0.0	23.8	84	100	92	61	71	47
III...	7.0	56.2	0.1	34.5	81	100	92	63	71	44
IV...	5.1	43.3	1.5	13.6	116	86	68	22	46	23
V...	5.1	52.9	10.5	12.0	91	83	69	26	45	17
VI...	5.0	62.3	18.3	12.7	127	82	70	25	44	15
VII...	5.1	47.9	0.9	19.7	113	86	71	29	49	26
VIII...	5.3	56.4	2.9	26.9	108	89	76	37	50	28

from 99 to 46 or 30, have shown no influence upon the rapidity of the processes of decomposition.

A more detailed report of these investigations will be published in the *Annals of the Agricultural College of Sweden.*

AMMONIFICATION OF DICYANODIAMIDE AND ITS DERIVATIVES IN SOIL

H. MURATA

Kagoshima Imperial College of Agriculture & Forestry, Kagoshima, Japan

IN THOSE countries which have hot summer and heavy rainfall, rice is the most suitable grain crop. In eastern Asia and in India rice has been first on the list of grain crops for many years. It is grown mostly under irrigation. In the principal districts of Japan, owing to a mild winter and the very intensive cultivation, the land is farmed on a well established system of *multi-crops*. Rice is grown every year on the same field as the summer crop. During the vegetative period of the rice, the soil is submerged or water-logged for nearly four months. The season of rice culture is followed by a season of a winter crop; during this season, the land is not irrigated and the soil is under normal aerobic conditions.

There has been inadequate distinction between ordinary soil conditions and those where the soil is water-logged, and this has led to erroneous assumptions concerning water-logged soils, particularly of rice fields.[1] In earlier work on the changes of cyanamide in the soil the present writer made a comparative study with normal dry-field and water-logged paddy-field conditions.[2] The results showed that the formation of ammonia from cyanamide, mainly through urea, is nearly the same under both soil conditions, namely the normal aerated soil and the water-logged soil. The decomposition of dicyanodiamide and guanylurea in water-logged soil is, however, quite different from that in normal soil. Previous studies of these changes by the writer in Japan[3] and in England[4] have shown that in water-logged soil dicyanodiamide and guanylurea are fairly readily ammonified, especially at warm temperatures, such as the summer months in Japan when the temperature of the surface water which covers the rice fields is about 30-40°C. Numerous laboratory and field experiments have already been reported which deal with the decomposition of dicyanodiamide in soil and in culture media. From these it has been concluded that, in normal soil, dicyanodiamide is slowly converted into ammonia and that it acts harmfully, mainly indirectly, preventing nitrification. On the other hand it has been found that, in Japan, dicyanodiamide and guanylurea are good fertilizers for paddy-rice and rush (*Juncus decipiens* Nak.).[2, 5, 6] The definite fertilizing value of dicyanodiamide on paddy-rice, as

contrasted with its harmful effect on most plants, was explained by the writer as due to the following effects:[2]

1. Under the water-logged condition of the paddy-soil in summer, dicyanodiamide is easily ammonified.

2. The indirect harmful effect of dicyanodiamide, by reason of its retardation of nitrification, does not affect the paddy-rice, since even without dicyanodiamide there is practically no nitrification in the water-logged paddy-soil, and nitrification is not essential in paddy-rice culture. Consequently, when using commercial cyanamide, the secondary harmful effects of the dicyanodiamide which may be formed from cyanamide, are apt to be entirely absent in paddy-rice culture, although they may be prominent in normal dry-field crop culture.

From the practical point of view, however, dicyanodiamide has little importance as a fertilizer, even for paddy-rice. Owing to the fact that dicyanodiamide is not readily absorbed by the soil, it is apt to be washed away by drainage water before undergoing ammonification by soil organisms. Thus dicyanodiamide does not show any significant fertilizing effect on paddy-rice in field experiments, although it shows remarkably good effects on plants in pot cultures.[7] Guanylurea, however, may be applied as a fertilizer for paddy-rice in the form of its salts, such as the sulphate and phosphate. Soil retains guanylurea better than ammonia.[3]

Recent studies by the writer at the Rothamsted Experimental Station in England,[4] revealed that anaerobic soil conditions created by other means than water-logging, such as the addition of reduced iron to the soil or placing soil in an atmosphere of hydrogen gas, also had a favorable effect upon the microbial ammonification of dicyanodiamide. It was also found that ammonification of dicyanodiamide in water-logged soil is greatly accelerated after the appearance of significant amounts of ferrous iron in the soil suspension. These facts suggest that an important factor for the microbial ammonification of dicyanodiamide in the soil is the low partial pressure of oxygen.

The writer continued his research at the Institut für Pflanzenernährungslehre und Bodenbiologie der Universität Berlin, in Berlin-Dahlem, Germany. The relation between the oxidation-reduction potential and the microbial decomposition of added dicyanodiamide and guanylurea sulphate in water-logged soil was particularly examined. Within the range of the experiments, the lower the redox-potential the more rapid was the ammonification of dicyanodiamide and guanylurea.[8] There was no appreciable ammonification of the above stated substances during a period of a few weeks until the Eh was below 250 mv. at pH 7.0. The main cause of lowering the redox-potential to the range favorable for the ammonification of dicyanodiamide and guanylurea, is the microbial decomposition of organic substances, contained in or added to the soil.

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A SCHEME OF PRELIMINARY INVESTIGATIONS PROPOSED FOR THE MICROBIOLOGICAL CHARACTERISTICS OF SOILS

J. MARSZEWSKA-ZIEMIECKA

National Institute for Agricultural Research, Pulawy, Poland

IN SPITE of the well-known difficulties connected with the evaluation of microbial activities in soils, the problem ought to be considered as very vital for an understanding of the general characteristics of soils. Recent progress of direct methods in soil microbiology tends to make this task easier. On the basis of these methods, the author suggests the adoption of a following scheme of investigations:

1. Qualitative observation on the distribution and growth cycle of micro-organisms on buried slides (Rossi-Cholodny), especially on slides coated with different substances (Ziemiecka).

2. Estimation of the density of population originally present in soils, using the ratio method of Thornton and Gray.

3. Quantitative evaluation of energy of growth of this population in soils, when added with a set of common sources of energy and nutrients. The ratio method has been successfully adapted to this purpose.

4. Study of CO_2 evolution from the soils.

5. More detailed study of the activities of organisms in elective cultures of Winogradsky; additional methods, when needed.

Special stress may be laid on the study of nitrogen fixation, cellulose decomposition, and the nitrifying power of soils, these processes being closely associated with general soil fertility conditions.

DIE BIOAKTIVITÄT DER WÜSTENBÖDEN

D. Fehér

Institute of Forestry, Sopron, Hungary

Infolge der extremen Verhältnisse des Wüstenklimas bildet die Erforschung der Lebenserscheinungen der Wüstenböden einen der interessantesten Probleme der mikrobiologischen Forschung. Die Erfassung der tatsächlichen Verhältnisse erforderte eingehende Untersuchungen an Ort und Stelle, da nur diese zu dem erwünschten Erfolg führen konnten. Wir haben mit Prof. Killian im Jahre 1934 und im Jahre 1936 zwei mikrobiologische Expeditionen in die Sahara geführt, wobei die wichtigsten Hauptbodentypen von dem Sahara-Atlas über die trockensten Wüstengebiete und über dem Hoggargebirge bis zu dem Savannen des Sudans biologisch und biochemisch untersucht worden sind.

Wir haben hierbei durch direkte und indirekte Methoden das tatsächliche Vorhandensein von lebenden Mikroorganismen untersucht und dabei auch die biochemischen Eigenschaften dieser Böden erforscht. Diese Forschungen haben zunächst den einwandfreien Beweis geliefert dass die Südsahara, von Mikroorganismen bewohnt sind, die dort, trotz dem enormen Wassermangel und der enormen Überhitzung dieser Böden, ein aktives Leben führen können.

Wir haben zunächst mikrobiologische Bodenatmung konstatiert und eine Reihe von Bodenbakterien, darunter viele Aktynomyceten, weiter Bodenpilze und auch Bodenalgen nachweisen können. Diese Resultate bestätigen also, dass die Grenzen des Mikroorganismenlebens viel weiter gezogen sind, als man sie früher vorausgesetzt hat und beweisen auch die extreme Anpassungsfähigkeit der dort gefundenen Formen. Sie haben ausserdem klargemacht, dass die bisher gemeinte Sterilitat und Inaktivität der Wüstenböden nicht vorhanden ist.

THE INFLUENCE OF REACTION UPON THE DEVELOPMENT OF AN ACID-TOLERANT AZOTOBACTER

ROBERT L. STARKEY

New Jersey Agricultural Experiment Station, New Brunswick, New Jersey, U. S. A.

EVER since the aerobic, nonsymbiotic, nitrogen-fixing bacteria were first cultivated there has been considerable interest in the tolerance of the organisms to acidity. In his initial report on the isolation of *Azotobacter*, Beijerinck recorded that in media to which KH_2PO_4 was added in place of the K_2HPO_4 usually added, the acid reaction was unfavorable to development of the organism and there was no growth.[1] As shown by Lipman,[16] factors affecting the reaction of the media led Beijerinck to conclude that *Azotobacter* fixed nitrogen only when grown in association with certain other bacteria.[1, 2]

The results gathered during the last few decades on the effect of reaction are too well known to require detailed elaboration. Christensen[3, 4, 5, 6, 7] used *Azotobacter* in a method for determining the reaction of soils and found a close correlation between the occurrence of the organism and the lime requirement of the soils. Gainey likewise found that *Azotobacter* could seldom be recovered from soils more acid than pH 6.0.[12, 13, 14, 15] From physiological studies with static cultures and by means of the Warburg technic, Burk, Lineweaver, and Horner[8, 9, 10, 11] were unable to obtain development of *Azotobacter* in nitrogen-free media below reactions close to pH 6.0. It was noted, however, that the cultures developed at somewhat more acid reactions in the presence of available fixed nitrogen compounds, and the conclusion was reached that, in cases where *Azotobacter* develops in soils more acid than pH 6.0, it is likely that the organism depends upon fixed nitrogen to supply its requirements for the element.[11]

So much evidence has been gathered by the aforementioned investigators and by numerous others to the effect that all species of *Azotobacter* are sensitive to acid reactions that it seems likely that there would be rather general agreement to the following statement by Burk and associates:[11] "The limit of fixation at pH 6.0 has been universally observed by all investigators."

DISTINCTIVE CHARACTERISTICS OF AZ. INDICUM

Almost invariably, media used for the cultivation of *Azotobacter* are neutral or somewhat alkaline in reaction, and an excess of $CaCO_3$ is present in

many such media. It was therefore a surprise to find that $CaCO_3$ exerted unfavorable effects on development of the species of *Azotobacter* considered in this report.

This organism, *Az. indicum*, was isolated from acid rice soils (pH 4.9-5.2) of India by De. As recently reported,[17, 18] it differs in several respects from the well-known species of the genus. The cells are motile and are somewhat oval to elongated. Under most conditions they are smaller than the cells of other *Azotobacter*, the size being 0.5-1.2x1.7-2.7μ in contrast to that of cells of other species of *Azotobacter*, which generally measure 2-3μ or more in breadth and up to 5 or 6μ in length. In almost all cases the cells are characterized by the presence of large fat globules, generally one large one at each end. These globules extend entirely across the cells at the terminal regions and may occupy more than one half of the total cell volume. Recently, some cultures of the organism were obtained with cells lacking these fat globules; they had the granular appearance more characteristic of the common *Azotobacter*. No particular cultural conditions were noted to which the change could be ascribed; the cultures were obtained by picking colonies from plates prepared from the cells containing the globules.

Another distinctive characteristic of the organism is its production of slime. Agar surfaces become covered with a profuse, raised, starchlike mass of cell material imbedded in slime which is commonly very elastic. Fluid media may become somewhat viscous, particularly during relatively short periods of incubation. As growth progresses in culture solutions, turbidity becomes uniform and dense and considerable sediment forms. Commonly a ring of cell material accumulates at the region of contact of the glass and the surface of the liquid. Occasionally a mucoid membrane spreads over the entire surface of the medium, but more commonly no pellicle is formed.

The organism grows particularly well upon sucrose and glucose but develops poorly upon mannitol. In all cases the organism grows less rapidly than other species of *Azotobacter*. Whereas good development appears on agar slants in a few days, maximum fixation of nitrogen in fluid media requires a period of several weeks.

DEVELOPMENT ON NITROGEN-FREE AGAR SLANTS

In the first tests of the influence of reaction upon development of the organism the following agar medium was used: distilled water—1000 cc., glucose—15.0 gm., K_2HPO_4—0.8 gm., KH_2PO_4—0.2 gm., $MgSO_4.7H_2O$—0.2 gm., NaCl—0.2 gm., $CaCl_2.2H_2O$—0.05 gm., $Na_2MoO_4.2H_2O$—0.0005 gm., $MnSO_4.4H_2O$—0.0005 gm., $Na_2WO_4.2H_2O$—0.0005 gm., $FeSO_4.7H_2O$—0.025 gm., agar—20.0 gm.

Varying amounts of 0.05 *N* HCl or 0.1 *N* Na_2CO_3 were added to 10-cc. portions of the medium in test tubes, and a series having a wide range of

reactions was thus prepared. The acid, alkali, and glucose were sterilized separately from the rest of the medium. The Na_2CO_3 was sterilized by passage through a Seitz filter. Two inoculated and one uninoculated agar slant were prepared at each reaction level. After incubation, the reaction of each tube was determined by means of the glass electrode. In preparing the suspensions, 5 cc. of distilled water was added to each tube of agar, and the cell material and agar were broken up and mixed with the water. The determinations were made after the mixtures had stood for about two hours with occasional stirring. Some of the results are summarized in table 1.

TABLE 1

DEVELOPMENT OF AZ. INDICUM ON AGAR SLANTS AT VARIOUS REACTIONS*

Reaction (pH)		Evidences of growth†	Consistency of cell material
Uninoculated	Inoculated		
9.0	7.6	+++B	
8.9	7.3	+++B	
8.7	7.2	+++B	Mucoid, slimy
8.1	6.8	+++B	
7.3	6.5	+++B	
6.8	6.2	+++B	
6.5	6.1	+++B	
6.2	5.6	+++LB	Slimy, ropy
5.8	5.4	+++C	
5.5	4.7	+++C	
4.5	4.2	+++C	Tenacious
4.0	3.8	+++C	
3.9	3.5	+++C	Gummy, rubbery, elastic
3.5	3.2	+++C	Very tenacious, rubbery
3.1	3.1	++C	
3.1	3.1	....	....

* Incubation 28 days. Averages of duplicate tubes of inoculated media.
† B-buff, LB-light buff, C-colorless.

Growth was evident at all reactions from pH 9.0 to and including 3.1. The organism failed to develop on only one of the media, that having an initial reaction of pH 3.1. In virtually all the media, even the very acid ones, the reaction became even more acid during growth; the changes were most marked in the media that were initially alkaline. The changes in pH in response to growth are somewhat less apparent than real, since a considerable portion of the agar slant which was not in contact with the cell material must have been affected relatively little during growth.

Below pH 4.0 there was relatively little cell material during the first few

days, but, on prolonged incubation, the organism grew well on all media except the one mentioned above. During the first week there was no evidence of pigmentation, but there was subsequently a buff to rust-brown color in the cultures growing on the alkaline and slightly acid media, the color being confined to the surface of the cell material. With the more acid media there was no evidence of any color even though the cultures were incubated for more than a month.

The consistency of the cell material varied with the reaction of the medium, but in every case slime was abundant. On the alkaline media the cell material was more watery and of thinner consistency than that on the acid media; during incubation, much of it accumulated at the base of the slant. At high acidity (initial pH below 4.0) the cell material was very tenacious, rubbery, and gummy, and considerable difficulty was encountered in removing any portion of it from the slant; it showed no tendency to settle to the base of the slant but clung together and could all be moved on the agar surface as a single mass. Between pH 4.0 and 6.0 the consistency was intermediate between the firmer gummy mass and the thin slime of the alkaline media. It was very mucoid, somewhat elastic, and could be drawn out in long threads with the transfer wire. The consistency of the slime became somewhat thinner as the incubation period was prolonged.

There was less evidence of change in the morphology of the cells than in slime production. At all reactions the cells were rod-shaped and characterized by the occurrence of fat globules. In most of the cells there were two globules, one at each end. With increasing acidity the cells were generally larger, and below pH 4.0 many were one and one-half to two times the size of the cells in media near neutrality. It was not uncommon to find such cells with three or even more fat globules, one at each end and the third near the center of the cell and extending completely across its short dimension.

From these observations it is apparent that *Az. indicum* is extremely tolerant to extreme acidity and develops well over a wide range of reaction. The limiting acidity appears to be close to pH 3.1. The alkaline limit is unknown, since in the medium used it was not possible to raise the pH above 9.0. Even though the reaction was initially adjusted to pH 10.0 and above, it dropped in the uninoculated media to 9.0 upon standing. The most striking changes in response to changes in reaction were modification in the characteristics of the slime and relatively minor alterations in cell size. It is perhaps surprising that there were such slight changes, in view of the fact that *Azotobacter* undergoes such pronounced morphological changes when certain substances are incorporated in the media. It is still more remarkable, however, that there should have been any development at all under the extremely acid conditions.

DEVELOPMENT ON AGAR SLANTS CONTAINING AMMONIACAL AND NITRATE NITROGEN

Previous observations had shown that some organic nitrogenous materials were unfavorable to development of the organism.[18] On the other hand, various other species of *Azotobacter* develop well upon both ammoniacal and nitrate nitrogen. Agar slants were therefore prepared having the same basic composition as the medium listed above. Some tubes received $NH_4H_2PO_4$ and others $NaNO_3$ in 0.1 per cent concentrations.

Development was good in the presence of both sources of nitrogen, but differences related to the added nitrogenous materials were marked. In the medium with nitrate the pH increased from the initial 6.5 to 7.7, as would be expected from the selective absorption of nitrate. The consistency of the cell material was mucoid and slimy but of the watery type associated with alkaline media. The cells were typical in appearance. In the presence of ammoniacal nitrogen the medium became distinctly acid. The pH dropped from 6.5 to 3.5, and the cell material had the characteristic rubbery and tenacious consistency common to acid media. These results suggest that *Az. indicum* utilizes both ammoniacal and nitrate nitrogen. Whereas there were noticeable differences in the characteristics of the slime caused by the reaction changes associated with the selective absorption of the nitrogenous materials, the globules of fatty materials did not disappear from the cells.

DEVELOPMENT IN NITROGEN-FREE FLUID MEDIA

The usual glucose medium was used after being adjusted to various reactions with 0.1 *N* HCl or 0.5 *N* Na_2CO_3. The glucose, HCl, and Na_2CO_3 were sterilized separately. The medium was used in 100-cc. amounts in 500-cc. Erlenmeyer flasks. Although the initial reaction of the most alkaline medium was pH 10.6 it dropped to 8.9 in the uninoculated control. As shown in table 2, *Az. vinelandii* developed only in the media having reactions above pH 6.0, but in no case did it appreciably alter the reaction of the medium. *Az. indicum* developed in all the media, even in the most acid one having an initial reaction of pH 3.0. In almost all cases, a pronounced increase in acidity accompanied growth. Much the same amounts of nitrogen were fixed in media having initial reactions of pH 3.1 to 8.9. Relatively little nitrogen was fixed in the most acid medium, pH 3.0. Even here, however, there were definite growth and significant fixation. Even though *Az. vinelandii* fixed large amounts of nitrogen, it did not surpass the fixation by *Az. indicum*. *Az. vinelandii* developed more rapidly and would have shown evidence of greater fixation than *Az. indicum* if the incubation period had been short.

TABLE 2

DEVELOPMENT OF AZ. INDICUM AND AZ. VINELANDII IN NITROGEN-FREE DEXTROSE SOLUTION*

Uninoculated pH	*Az. vinelandii*		*Az. indicum*	
	pH	N fixed†	pH	N fixed†
		mgm.		*mgm.*
8.9	8.8	8.5	7.9	13.9
8.5	8.6	10.0	7.6	13.5
8.3	8.3	10.5	7.0	9.9
7.7	7.8	12.6	6.4	10.9
7.1	7.1	12.0	5.8	10.5
6.9	6.9	13.5	5.3	12.4
6.6	6.5	14.0	4.9	12.7
6.2	5.9	13.6	3.9	13.9
4.9	4.9	0	3.6	11.4
3.7	3.7	0	3.4	7.9
3.3	3.3	0	3.1	11.3
3.1	3.1	0	3.1	11.6
3.0	3.0	0	2.9	3.4

* Incubation period 70 days. Averages of duplicates of inoculated cultures.
† Results reported with uninoculated controls subtracted. Average of controls 1.7 mgm. N.

DEVELOPMENT IN FLUID MEDIA CONTAINING FIXED NITROGEN

Four types of solution media were prepared having the basic composition of the medium given previously. One series of 500-cc. Erlenmeyer flasks each containing 100 cc. of the solution was left without nitrogen. To the second series was added $(NH_4)_2SO_4$, to the third $NaNO_3$, and to the fourth urea. The amounts of the nitrogenous materials added to the solution in each flask contained approximately 25 mgm. of nitrogen. The media were all virtually neutral at the start. After incubation for periods of 25 and 80 days, determinations were made for various nitrogenous constituents and for reaction. The results reported in table 3 are typical of those obtained with three different cultures of *Az. indicum*.

In the medium to which no nitrogen was added, the pH dropped slightly as the organism developed, and considerable nitrogen was fixed. During the longer incubation period this amounted to about 11 mgm. of nitrogen fixed per gram of carbohydrate added to the medium.

The reaction became extremely acid in the inoculated medium to which the ammonium sulfate had been added, dropping from the initial reaction which was close to neutrality to pH 3.4. Considerable amounts of the ammoniacal nitrogen were assimilated. The fact that less nitrogen was absorbed than was

TABLE 3

DEVELOPMENT OF AZ. INDICUM IN MEDIA CONTAINING FIXED NITROGEN

Nitrogen source	Incubation period	Treatment	Reaction pH	Nitrogen balance			
				Nitrate N	Ammonia N	Urea N	Total N
	days			*mgm.*	*mgm.*	*mgm.*	*mgm.*
None....	25	Uninoculated	7.1	0	0	..	0.8
	80	Uninoculated	7.1	..	0	..	1.3
	25	Inoculated	6.7	0	0	..	5.0
	80	Inoculated	6.4	..	0	..	18.1
Ammonia.	25	Uninoculated	6.9	0	22.3	..	23.0
	80	Uninoculated	6.6	..	21.8	..	..
	25	Inoculated	3.4	0	16.0	..	23.4
	80	Inoculated	3.4	..	16.5	..	25.3
Nitrate...	25	Uninoculated	7.2	23.5	0	..	24.3
	80	Uninoculated	7.1	23.5	0	..	24.1
	25	Inoculated	7.4	13.8	0	..	26.0
	80	Inoculated	8.3	6.5	0	..	24.9
Urea.....	25	Uninoculated	7.1	..	0	22.8	24.5
	80	Uninoculated	7.2	..	0	22.5	25.1
	25	Inoculated	8.0	..	10.5	0	15.1
	80	Inoculated	7.9	..	6.5	0	9.7

fixed in the absence of nitrogen addition during the longer incubation period may be due to the inhibitive effect of the high acidity. Virtually no change seems to have occurred between the twenty-fifth and the eightieth days of incubation.

In the presence of nitrate the pH increased, particularly during the longer incubation period. About 40 per cent of the nitrate nitrogen was assimilated in 25 days and 70 per cent in 80 days, the amount being practically the same as that fixed in the nitrogen-free medium in 80 days.

The medium containing urea did not support appreciable growth. Since the organism is able to utilize atmospheric nitrogen and, furthermore, since all of the urea had been decomposed and there was ammoniacal nitrogen in the inoculated medium, lack of available nitrogen could not be responsible for the inhibition. The medium was distinctly alkaline at the first incubation period and may have been even more alkaline during the first few days of cultivation. At least the medium was sufficiently alkaline so that about 40 per cent of the total nitrogen was lost in 25 days and 60 per cent in 80 days; this loss can be ascribed to the volatilization of ammonia from the alkaline

liquids. It seems reasonable to assume that some intermediate nitrogenous product of urea breakdown limited development.

As a whole, the results verify the observations made on solid media and indicate that *Az. indicum* is able to assimilate both ammoniacal and nitrate nitrogen. It is further evident that nitrogen fixation is inhibited by the presence of available fixed nitrogen.

DISCUSSION

From this brief discussion of the tolerance of *Az. indicum* to extremes of reaction it is apparent that this organism is not only able to grow at far greater hydrogen-ion concentrations than any previously reported aerobic nitrogen-fixing bacterium but it is able to fix nitrogen under these conditions. This suggests the possibility that *Azotobacter* may be active in some acid soils previously assumed to be unable to support development of these organisms. Whether or not organisms similar to the one discussed are widely distributed still remains to be ascertained. Burk and associates[8, 10, 11] concluded that one of the factors affecting the specific nitrogen-fixing enzyme system of *Azotobacter* is hydrogen-ion concentration and that concentrations greater than 10^{-6} inactivate the system. These conclusions were based upon measurements of the medium in which the organism was cultivated. The results contained in this report might suggest that the enzyme system of *Az. indicum* is different from that of the other species of *Azotobacter*, but there is no real justification for this assumption. The possibility also exists that the liquid within the cell at regions where nitrogen fixation may be assumed to occur has quite different characteristics from that of the external medium. This being the case, it may be unjustified to assume, in the absence of specific information, that the hydrogen-ion concentration at the seat of nitrogen fixation differs with the acid-tolerant and acid-sensitive species of *Azotobacter*.

SUMMARY

Azotobacter indicum Starkey and De, has been found to develop over a wide range of reactions. Both on agar and in liquid media it grew in the absence of fixed nitrogen at pH values from 9.0 to nearly 3.0. On solid media the nature of the slime, which is always produced in abundance, was altered with changes in reaction; in alkaline media the slime was somewhat watery, whereas it became tough and rubbery in very acid media. The media consistently became more acid during growth. The organism assimilates both ammoniacal and nitrate nitrogen and hydrolyzes urea to ammonia but made limited development in the medium containing urea. Nitrogen fixation was inhibited in the presence of these fixed forms of nitrogen. During growth of the organism in media containing ammonium salts the reaction became

very acid. The reaction became somewhat more alkaline where nitrogen was supplied as nitrate. These changes are the result of the selective absorption of the ammonium and nitrate nitrogen.

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THE EFFECT OF BENZOIC ACID COMPOUNDS UPON THE ABUNDANCE OF MICROORGANISMS, INCLUDING AZOTOBACTER ORGANISMS, IN A SOIL

HERBERT W. REUSZER

Colorado Agricultural Experiment Station, Fort Collins, Colo., U. S. A.

THE fact that sodium and calcium benzoate may be utilized by *Azotobacter* as sources of energy was noted by Winogradsky[4] a number of years ago. The effect of compounds of this type upon the general microbial population of a soil or upon its *Azotobacter* population appears never to have been studied. Because of their known bacteriostatic action upon many organisms, it would be expected that these compounds when added to soil might possibly upset the normal balance of the soil population in favor of the *Azotobacter* organisms. To test the effect of benzoate compounds, the experiments described below were set up. It was found that the compounds brought about great increases in the abundance of soil microorganisms and especially of *Azotobacter* organisms. Of greater interest, however, was the observation that in soils receiving high treatments of sodium benzoate or benzoic acid the *Azotobacter* population consisting normally of *Az. chroococcum* and *Az. beijerinckii* was displaced completely by a green-pigment-producing form of *Azotobacter* which had not before been found in this soil.

EXPERIMENTAL

Methods. Sodium benzoate, benzoic acid, and calcium benzoate were added to soil in quantities equalling 1.0, 2.5, and 5.0 per cent of the air-dry soil. Untreated samples were included for comparison, and all treatments were duplicated. Moisture content was kept at 20 per cent of the air-dry weight of soil. Incubation was at 28°C. After 1, 3, 5, 7, 9, 12, and 15 weeks of incubation, samples were taken for the following determinations: abundance of bacteria and actinomyces, mold fungi and *Azotobacter* organisms, pH, and nitrate nitrogen content. The counts of microorganisms were all made by the plate method, using standard media. It was expected that separate counts of bacteria and actinomyces would be made on the same plates. It was found, however, that on plates from most of the treatments

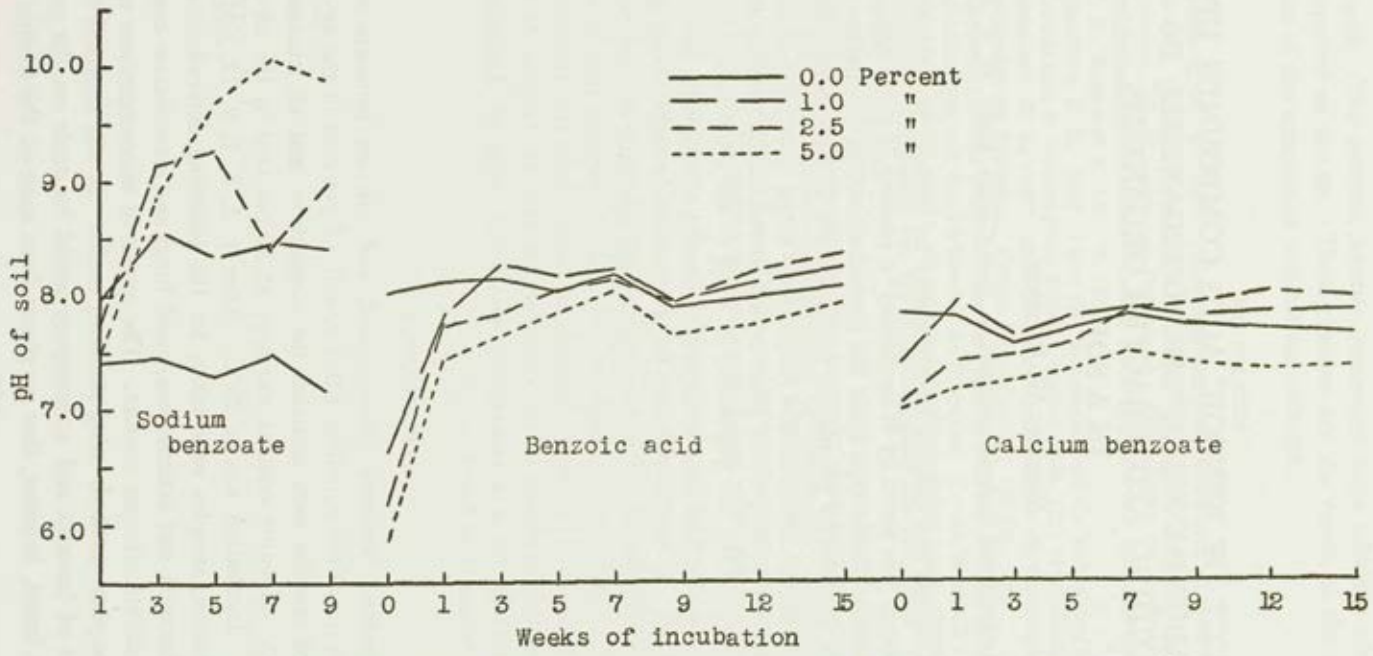

FIG. 1. pH values found in soils incubated with and without added benzoic acid compounds.

the two groups of colonies could not be distinguished without microscopic examination of a majority of the colonies on each plate. Since this would have required a prohibitive amount of time, only the combined values for these two groups of organisms are given.

Effect upon pH. With compounds such as those used in this experiment, the effect upon the microflora was not due simply to addition of available energy material. There were, for example, considerable pH changes induced either directly upon addition of the compounds or during their subsequent decomposition. This effect was most pronounced in the case of sodium benzoate, as may be seen in figure 1. Unfortunately, no pH determination was made upon mixing the soil and sodium benzoate; consequently, the immediate effect of the benzoate is not known. The fact that after 1 week of incubation the pH of the soil receiving 5 per cent sodium benzoate, a quantity which was sufficient to delay the beginning of microbial activity, was not above that of the untreated soil indicates that the direct effect was small. Where smaller quantities of sodium benzoate were used, and early microbial activity was greater, the pH change was greater also. With an addition of 5 per cent of the compound, the pH value rose to a maximum of about 10 after incubation for 7 weeks. Where 2.5 per cent of the benzoate was added, the maximum pH of 9.3 was reached at 5 weeks' incubation. With 1 per cent sodium benzoate, the maximum, which did not exceed pH 8.6, was reached still earlier.

The addition of benzoic acid depressed the soil pH considerably, depending upon the amount added. After 1 week of incubation, the pH of all the treated soils had risen to a value slightly below that of the untreated soil, probably because of the combined effect of decomposition of the acid and its chemical combination with calcium carbonate or other basic substances in the soil. Following this initial rise, the pH values apparently followed a course similar to that in the untreated soil, being either above or below those found in the control soil, depending upon the amount of benzoic acid added. Calcium benzoate brought about the least change in soil pH. Decomposition of the compound brought about a pH higher than that in untreated soil, except for the 5 per cent addition. The pH changes will be discussed in more detail in connection with a consideration of the microbial changes. Any other possible effects due to the cations were not studied in this investigation.

Solubility of compounds. Because of its effect upon the osmotic pressure of the soil solution and the availability of the compounds to the microorganisms, the factor of solubility of the different compounds must also enter into any consideration of their effect upon the microbiological population. The solubility of sodium benzoate is such that, even with the largest amount added, all the compound must have become available in a soluble form to the soil microorganisms. On the other hand, with the low solubility of

benzoic acid, only a small portion of this substance would be available in solution. The same is true of calcium benzoate, although its solubility is considerably greater than that of benzoic acid. With these last two compounds, the size of particle would be expected to play a part, because of its relation to the amount of surface exposed to microbial attack. The calcium benzoate used was in a very finely powdered form; consequently, it had a much greater surface than did the benzoic acid, which was in crystalline form.

Abundance of mold fungi. The effect of the benzoate compounds upon the abundance of molds is shown in the lower part of Figure 2, where the logarithms of the numbers found are plotted. In the untreated soils the counts indicate numbers of molds much below those found in treated soils. At the 1-week incubation period, the number of molds in the three sodium benzoate-treated soils did not differ greatly; they were, however, in inverse ratio to the amount of benzoate added and were all above the number found in the control soil. Following this period, the determinations showed a rapid rise to very high levels in the soils to which were added 1 and 2.5 per cent of sodium benzoate. The increase in the 2.5 per cent treated soil lagged behind that in the 1 per cent treated soil until the fifth week of incubation, when the numbers became greater in the soil with the larger addition of the organic compound. The number of molds in the soil receiving 5 per cent sodium benzoate remained much below that found in the other treated soils but much above that in the untreated soil. This may be ascribed to a greater toxic action of such a high concentration of benzoate and to an increase of pH to a level so high as to be detrimental. The toxic action probably decreased as more of the benzoate was decomposed; but the high pH apparently limited the fungal growth, since the maximum number was reached at the seventh week of incubation at a level much below that in soils receiving smaller quantities of benzoate.

The changes in mold population of the benzoic acid-treated soil bear a general resemblance to those described for sodium benzoate-treated soils. The 5 per cent benzoic acid treatment appeared to exert a more toxic action than did a similar treatment of sodium benzoate, since it reduced the number of molds at the first week of incubation to a point below that found in the untreated soil. With benzoic acid, the lag in increase of molds in the 2.5 and 5 per cent treatments was more pronounced than was the case with sodium benzoate.

The addition of calcium benzoate brought about changes in the mold population differing considerably from those found for the other treatments. With 1 per cent calcium benzoate, the number of molds only slightly exceeded that present in the control soil, and the number present after 1 week of incubation was already almost equal to the maximum number found during the entire period. Larger additions of calcium benzoate gave increases almost

FIG. 2. Effect of benzoic acid compounds upon the abundance of microorganisms in a soil.

parallel until the ninth week, after which the number in the 2.5 per cent treatment continued to increase, while a decrease took place in the 5 per cent treatment.

Abundance of bacteria and actinomyces. The logarithms of the numbers of bacteria and actinomyces are plotted in the upper part of Figure 2. The course followed by numbers of these organisms in the various sodium benzoate-treated soils was similar to that described for mold fungi. Much greater numbers of bacteria and actinomyces than of molds were present. The toxic action of the compound appeared to be more pronounced upon this group of organisms, since both the 2.5 and 5 per cent treatments depressed numbers below those in the control soil at the initial period of incubation. This apparently was also the case with benzoic acid, where such an action may have been intensified by the originally low pH. The subsequent rise in numbers was less marked in benzoic acid-treated soils than in those receiving sodium benzoate. In both cases, the maximum number of organisms in the lower rates of treatment was reached early in the incubation period; whereas with the 5 per cent treatment the maximum was not reached until the end, and the number still appeared to be increasing. These data would indicate that where the smaller quantities of benzoate were added their decomposition was almost complete by the end of 3 weeks' incubation but that the decomposition of the 5 per cent addition was still not complete after 9 or 15 weeks' incubation. For sodium benzoate, the same conclusion is reached from an examination of the pH data.

The lower concentrations of calcium benzoate brought about a more rapid increase in numbers of bacteria and actinomyces than did the other compounds. Thus, when 1 per cent calcium benzoate was used, the maximum number of organisms was found after only 1 week of incubation; and at subsequent periods their number dropped to a level little higher than that in the untreated soil. With the 2.5 per cent treatment, the number found at the initial period was maintained with little change throughout the period of incubation. In only these two treatments was there any evidence that the maximum number of microorganisms might have been reached before the first determination of abundance was made.

In these experiments, it was not possible to make any study of the types of organisms occurring in the treated soils. It was observed on the sodium albuminate agar plates, however, that with the higher treatments fewer types of colonies appeared and that this became more pronounced with increasing length of incubation period. This was particularly true in the case of the molds. Here one type of colony became the predominant form on plates from the treated soils; and since it could be readily identified, its numbers were recorded separately from the total count. The proportion of the total mold population made up by this organism, apparently an *Aspergillus* species,

is shown in table 1 for the benzoic acid- and calcium benzoate-treated soils. One per cent and 2.5 per cent benzoic acid brought about the almost complete elimination of other forms of molds appearing on the agar plate. With calcium benzoate, the organism made up a smaller proportion of the total molds present. Here the organism was most abundant on plates from the 2.5 and 5 per cent treated soils, and much less so in those from the 1 per cent treatment.

TABLE 1

PERCENTAGE OF TOTAL MOLD POPULATION OCCURRING AS A SINGLE SPECIES

Percentage of compound added to soil	Benzoic acid							Calcium benzoate						
	Weeks of incubation							Weeks of incubation						
	1	3	5	7	9	12	15	1	3	5	7	9	12	15
0.0.....	0	2	4	0.5	2	0	0	0	0	0	0	0	0	0
1.0.....	87	97	94	97	96	92	95	0	0	14	13	21	10	7
2.5.....	82	92	87	72	84	95	97	0	0	42	48	55	80	77
5.0.....	35	66	74	56	67	85	69	0	0	34	47	47	57	56

Abundance of Azotobacter organisms. The *Azotobacter* counts obtained in the variously treated soils are shown in table 2. The numbers found in the untreated soils were very low but were typical of those found in many similar determinations made in this laboratory. They were also found to be within the range of numbers found in many field soils examined. It may be seen that the addition of 1 per cent of any of the benzoic acid compounds brought about a tremendous increase in the abundance of *Azotobacter*. The increases were largest with this concentration of sodium benzoate, where they were so unexpectedly large that platings at the first three periods were not made with sufficiently great dilutions of the soil. Very much lower numbers were found where larger amounts of sodium benzoate were added. This no doubt was due both to a toxic effect of the high concentrations of benzoate and to the high pH values reached in these soils. Thus, in the 5 per cent treated soil the pH value reached a value higher than the maximum at which *Azotobacter* would be expected to be active, as indicated by the results of Burk, Lineweaver, and Horner.[1]

With benzoic acid, very large increases were also noted, although the *Azotobacter* determinations never indicated values as high as were found with the 1 per cent sodium benzoate treatment. With the 2.5 and 5 per cent benzoic acid treatments, the development of *Azotobacter* was retarded; but

TABLE 2

EFFECT OF BENZOIC ACID COMPOUNDS UPON THE NUMBER AND SPECIES OF *Azotobacter* ORGANISMS IN SOIL

(Number per gram moist soil)

Percentage of compound added	Weeks of incubation						
	1	3	5	7	9	12	15
Sodium benzoate							
0.0.....	60	157	64	63	32	....	....
1.0.....	>10,000	>100,000	>100,000	12,350,000	9,400,000	....	....
2.5.....	10	1,240	60*	482*	177*	....	....
5.0.....	60	8	1*	2*	1*	....	....
Benzoic acid							
0.0.....	100	78	87	73	94	57	210
1.0.....	>10,000	5,600,000	3,320,000	4,180,000	3,680,000	3,660,000	2,610,000
2.5.....	>1,000 14	120	>4,000*	>100,000*	2,990,000*	1,370,000*	730,000*
5.0.....	0	4	3*	50*	220* 5,500*	420* >100,000*	400* >1,000,000*
Calcium benzoate							
0.0.....	29	22	33	18	44	10	50
1.0.....	560,000	640,000	420,000	440,000	277,000	173,000	219,000
2.5.....	0	2 0	28 0	0 0	360 0	>1,000 >1,000	324,000 1,550,000
5.0.....	0	0	0	0	0	17	582 8

* The starred figures indicate samples in which the usual *Azotobacter* flora, consisting of *Az. chroococcum* with perhaps a small proportion of *Az. beijerinckii*, was completely or almost completely displaced by a green-pigment-forming strain. This strain was never observed in any other samples than those indicated.

In a few cases, widely divergent values were found in duplicate pots receiving the same treatment. In such cases, the values for both samples are given.

after a preliminary period of incubation, the values here also rose to very high numbers. This apparently did not take place until sufficient benzoic acid had been decomposed to permit multiplication of *Azotobacter*. This phenomenon was noted also with the high treatments of calcium benzoate, where the retardation period was rather long but where the values in soil with 2.5 per cent calcium benzoate finally exceeded those with 1 per cent of the compound.

It is obvious that the addition of the benzoic acid compounds increased the *Azotobacter* population to a point where it represented an appreciable part of the total bacterial population as determined by the plate method. It may be noted further that the number of *Azotobacter* in many cases exceeded the number of mold fungi found by the plate method. In no case did the *Azotobacter* organisms become the predominant form present, although they became so abundant as to appear frequently on the sodium albuminate agar plates used in determining the total number of bacteria and actinomyces.

Of even greater interest than the quantitative change brought about in the *Azotobacter* population by benzoate applications is the qualitative change indicated in table 2. As shown there, the 2.5 and 5 per cent treatments of sodium benzoate and benzoic acid brought about an *Azotobacter* flora consisting almost entirely of a green-pigment-producing form. In all other treated and untreated soils, the only *Azotobacter* colonies appearing were black-pigmented forms commonly associated with the species *Az. chroococcum*, and perhaps occasionally colonies of *Az. beijerinckii*. The fact that the green-pigment-forming organism appeared only after 3 to 5 weeks' incubation would seem to indicate that this form was present only in extremely small numbers in the soil used. It would seem further from these results that this organism is better able to develop in the presence of large amounts of benzoic acid compounds than is *Az. chroococcum*. This conclusion is supported by the finding[3] that the green-pigmented strain is capable of growing in the presence of considerably higher concentrations of sodium benzoate in artificial media than is *Az. chroococcum*. On the basis of studies not yet complete, it appears that the green-pigment-producing form is similar to others which have been identified as *Az. vinelandii*.[2] Winogradsky[3] has recently indicated the rarity and relatively unknown distribution of this particular organism. It would seem that this method of incubating soil with benzoic acid or sodium benzoate may have possible applications as a means of studying the ecological distribution of the green-pigment-forming strain of *Azotobacter*. Studies concerning this point are in progress.

SUMMARY

Sodium benzoate, benzoic acid, and calcium benzoate, when added to soil, caused large increases in the number of bacteria and actinomyces, mold fungi, and *Azotobacter* organisms as determined by the plate method.

Addition of 1 per cent of any of the compounds caused a more rapid increase than did additions of 2.5 or 5.0 per cent. The toxic effect of the larger additions was overcome as decomposition progressed and the concentration of particular substance decreased. This was not entirely the case with sodium benzoate, where the high pH induced by its decomposition appeared to limit microbial development, particularly that of molds.

Along with the increase in total number of microorganisms, the benzoic acid compounds caused a decrease in the variety of types appearing.

Sodium benzoate and benzoic acid, when used in concentration of 2.5 and 5.0 per cent, brought about the appearance of a strain of *Azotobacter* producing a green pigment. This organism was not found in any other treatments, nor had it ever been found previously in the soil used. The possible value of this finding in ecological studies of *Azotobacter* is indicated.

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SUR L'UTILISATION DES COMPOSÉS PHENOLIQUES COMME ALIMENT ENERGETIQUE PAR LES AZOTOBACTER DU SOL

G. GUITTONNEAU

Institut national agronomique de Paris

and R. CHEVALIER

Ecole nationale d'agriculture de Grignon

LA QUESTION ÉTUDIÉE

DANS deux mémoires publiés en 1926 et 1932, Winogradsky a apporté des éclaircissements sur les conditions qui régissent l'activité des Azotobacter dans le sol. En étudiant la question des sources d'énergie utilisables par les fixateurs aérobies du sol, l'illustre bactériologiste a été amené à poser le problème de leur prolifération aux dépens des composés aromatiques ternaires. Dès ses premières recherches Winogradsky a montré que les benzoates constituent d'excellents aliments pour les Azotobacter.

Les recherches que, de notre côté, nous avons poursuivies ces dernières années nous ont permis d'établir que les composés phénoliques eux-mêmes n'échappent pas à l'attaque des Azotobacter du sol et que le carbone des noyaux benzéniques intervient dans l'énergétique des fixations d'azote aérobies.

LA MÉTHODE

Nous avons utilisé la méthode directe de Winogradsky en la complétant dans quelques cas par la culture pure. Les cultures sur milieu électif ont été faites dans des boîtes de Pétri de 200 mm., 100 mm. et 50 mm. de diamètre contenant respectivement des quantités de silicogel égales à 200 cc., 30 cc., et 7 cc. Les acides étudiés ont été utilisés sous la forme de sels de sodium. Dans tous les cas, les millieux ont été ajustés à la neutralité.

COMPORTEMENT DES AZOTOBACTER DU SOL SUR MILIEU ÉLECTIF AU SILICOGEL

Prolifération d'Azotobacter aux dépens du salicylate de sodium. Nos premières expériences ont porté sur le salicylate de sodium que nous apportions à la dose de 0.5 g. pour 200 cc. de silicogel.

Une centaine de grains d'une terre riche en Azotobacter (sur plaque mannitée) servaient à l'ensemencement et on l'examinait journellement au cours de la période d'incubation de 15 jours à laquelle on la soumettait. Le nombre maximum de grains fertiles pour 100 grains ensemencés, dénombrés dans les conditions que nous venons d'indiquer, était de 25 après 10 jours.

La terre étudiée renfermait donc des fixateurs d'azote aérobies capables d'utiliser l'acide salicylique, mais leur prolifération était nettement moins abondante et moins rapide qu'avec le mannitol et le benzoate de sodium qui, dans des conditions identiques, nous donnaient un pourcentage de grains fertiles sensiblement égal à celui des grains ensemencés.

On pouvait penser que les différentes souches ou races d'Azotobacter du sol sont inégalement adaptées à l'attaque et à l'utilisation de l'acide salicylique ou que la dose expérimentée n'était pas la plus favorable.

Influence de la dose. Une série de plaques de silicogel de 200 mm. de diamètre imprégnées de quantités croissantes de salicylate de sodium étaient ensemencées avec des terres de qualités culturales variées.

Sous des indices numériques qui les rangeaient dans l'ordre décroissant de leur fertilité, on les désignait par les lettres J ou C suivant qu'elles provenaient de jardins ou de champs cultivés.

Le pourcentage de grains fertiles comptés après 15 jours d'incubation à 30° C. au cours de l'un de nos essais, est inscrit dans le tableau 1.

TABLEAU 1

Matière énergétique (gr. pour 1 plaque)	Nombre de grains fertiles pour 100 grains ensemencés					
	J1	J2	J3	C1	C2	C3
Salicylate de sodium 1,5.....	6	3	..	0	0	0
Salicylate de sodium 1,0.....	39	5	..	2	0	1
Salicylate de sodium 0,5.....	66	62	1	25	13	2
Salicylate de sodium 0,25.....	86	75	2	53	30	1
Salicylate de sodium 0,125.....	82	54	2	48	44	9
Mannitol 2,0.....	100	100	45	100	96	48

On voit que: 1) un rapport existe entre la fertilité des sols étudiés et leur teneur en souches actives vis à vis de l'acide salicylique; 2) l'action activante du salicylate est d'autant plus grande que la dose employée est plus faible. Celle de 0,250 gr. pour 200 cc. de silicogel s'est montrée la plus favorable.

Influence de la structure moléculaire des acides monoxybenzoïques. Les résultats que nous venons de rapporter nous ont conduits à étudier le comportement des Azotobacter vis à vis des isomères de l'acide monoxybenzoïque: ortho, méta, para, d'une part, de l'acide benzoïque d'autre part.

Des plaques de silicogel de 200 mm. de diamètre, imprégnées des sels de sodium de ces acides, étaient ensemencées avec un certain nombre des terres précédemment étudiées.

L'acide ortho-monoxybenzoïque mis à part, nous n'avons pas observé pour les autres acides de différences tenant à la dose utilisée, le pourcentage de grains fertiles avec 0,1 gr., 0,2 gr., 0,5 gr., étant sensiblement le même.

Pour les plaques à l'acide méta-monoxybenzoïque aucune végétation notable d'Azotobacter n'est apparue dans le délai normal de quelques jours.

Pour les autres acides, des colonies d'Azotobacter commençaient à devenir nettement visibles autour de certains grains, à partir du deuxième ou troisième jour. Le nombre maximum de colonies était noté après un temps ne dépassant jamais une quinzaine de jours, souvent inférieur à 10 jours.

Le pourcentage de grains fertiles, dénombrés au cours de l'un de nos essais, est indiqué dans le tableau 2.

TABLEAU 2

Terres ensemencées	Plaques à 0,200 gr.			
	Benzoate	Monoxybenzoates		
		Ortho	Méta	Para
J1.....	95	89	0	93
J3.....	9	2	0	27
C1.....	98	48	0	98

Ce tableau montre que parmi toutes les souches d'Azotobacter présentes dans les terres étudiées un grand nombre pouvaient utiliser comme aliment le para-monoxybenzoate, aussi bien et quelquefois plus facilement (terre J3) que le benzoate.

Un nombre nettement moindre s'attaquait à l'ortho-monoxybenzoate et enfin aucune n'avait d'action notable sur le méta-monoxybenzoate.

Cas de l'acide méta-monoxybenzoïque. La stérilité observée sur l'acide méta-monoxybenzoïque était-elle due à une inaptitude de l'Azotobacter à proliférer aux dépens de cet acide ou à l'action toxique de ce dernier?

Deux séries d'expériences nous ont permis de répondre à cette question.

1) Des grains de la terre J1, étant d'abord maintenus pendant 13 jours à l'état d'inertie apparente sur plaques au méta-monoxybenzoate, puis transportés aseptiquement sur de nouvelles plaques à 0,200 gr. d'acide benzoïque se sont montrés fertiles sur ce nouveau milieu en proportion normale.

2) Les grains de la terre C1 ensemencés sur plaques de silicogel à 0,200 gr.

d'acide méta-monoxybenzoïque + 0,200 gr. d'acide benzoïque s'entouraient de colonies d'Azotobacter à peu près aussi régulièrement que les grains de la même terre ensemencés sur plaques au benzoate seul.

De ces observations, nous pouvions conclure que le méta-monoxybenzoate n'a aucune action toxique appréciable sur les fixateurs d'azote aérobies et qu'il n'a pour eux aucune valeur alimentaire.

Attaque des noyaux benzéniques et utilisation alimentaire du phénol par les Azotobacter du sol. Nos études sur les acides monoxybenzoïques nous amenaient à penser que les groupements carbonés des noyaux benzéniques devaient prendre place dans l'énergétique des fixations d'azote aérobies du sol. Pour l'établir de façon certaine il était nécessaire de mettre hors de cause les chaînes latérales de ces acides et d'obtenir un processus de fixation aux dépens du carbone nucléaire.

En conservant le processus expérimental que nous avions précédemment utilisé il nous a été possible d'établir que le phénol lui-même peut être attaqué par certaines souches d'Azotobacter du sol.

Deux de nos terres types, J1 et C1, étaient ensemencées sur boîtes de Pétri renfermant 200 cc. de silicogel et imprégnées de doses croissantes de phénol.

Le tableau 3 résume les résultats enregistrés à partir du 3ème jour, d'une incubation à 30°, en chambre humide, qui a été prolongée durant 15 jours.

TABLEAU 3

Phénol en grammes par plaque.....	0,05	0,075	0,1	0,2
Pourcentage de grains fertiles J1..	20	33	33	2
Pourcentage de grains fertiles C1..	33	37	36	0

On voit que la dose la plus favorable est voisine de 0,100 gr. pour 200 cc. de silicogel. Nous avons vérifié que la dose de 0,2 gr. n'était qu'inhibitrice et qu'une dose de 0,5 gr. était germicide.

Ces résultats rapprochés de ceux que nous avions obtenus avec les acides monoxybenzoïques montrent, que pour les terres étudiées, les souches d'Azotobacter capables de s'attaquer au phénol sont moins nombreuses que celles qui peuvent utiliser le mannitol, l'acide benzoïque, l'acide para- et même l'acide ortho-monoxybenzoïque.

Existence de races. Les différentes souches d'Azotobacter présentes dans les terres étudiées étant inégalement adaptées à l'attaque des corps phénoliques étudiés, il était intéressant de mettre en évidence l'existence de ces souches et de faire ressortir leurs caractères physiologiques.

Cette étude a été résolue par la technique du transport des grains de terre d'une plaque à une autre que nous avons déjà définie.

Nous nous adressions de préférence à des terres du type J3 et C1 qui sur

certaines plaques de nos silicogels présentaient une proportion assez importantes de grains stériles. La dose de substance utilisée était celle que nous avions reconnue comme étant la plus favorable, c'est à dire 0,200 gr. pour 200 cc. de silicogel. Nous opérions avec des boîtes de Pétri de 50 mm. de diamètre ensemencées avec 1 seul grain de façon à éviter les contaminations par les colonies voisines.

En reprenant par exemple sur les plaques à l'ortho-monoxybenzoate les grains restés stériles après une incubation de 15 jours et en les transportant sur des plaques au benzoate, où beaucoup d'entre eux se montraient fertiles, on obtenait facilement des souches actives vis-à-vis du benzoate et inactives vis-à-vis de l'ortho-monoxybenzoate.

En généralisant les essais de ce genre, nous avons pu finalement grouper les Azotobacter des terres J1, J3 et C1 en 5 types qui se différencient physiologiquement comme l'indique le tableau 4, où à titre de référence, on a indiqué également les résultats obtenus avec le mannitol.

TABLEAU 4

	Mannitol	Benzoate	Phénol	Monoxybenzoate		
				Para	Ortho	Méta
1er type.....	actif	actif	inactif	actif	actif	inactif
2ème type.....	actif	actif	inactif	actif	inactif	inactif
3ème type.....	actif	inactif	inactif	actif	inactif	inactif
4ème type.....	actif	inactif	inactif	inactif	inactif	inactif
5ème type.....	actif	actif	actif	actif	inactif	inactif

Les 5 types de souches ainsi définis ne sont peut-être pas les seuls qu'il serait possible de trouver au cours d'études plus poussées, mais ce qui précède suffit à montrer jusqu'à quel point est poussée l'adaptation des Azotobacter à l'attaque des différents composés benzéniques qu'ils peuvent utiliser comme aliments énergétiques.

ATTAQUE DIRECTE DES COMPOSÉS PHÉNOLIQUES PAR LES AZOTOBACTER EN CULTURE PURE

Pour démontrer que dans nos expériences les fixateurs d'azote aérobies avaient pu utiliser directement les composés phénoliques sans le secours d'aucune bactérie spécifique des phénols il était nécessaire d'avoir recours aux cultures pures.

La purification des souches a été obtenue sur gélose aux sels nutritifs additionnée comme matière énergétique, soit de 1,75 gr. pour 1000 d'acide monoxybenzoïque, soit de 0,5 gr. pour 1000 de phénol.

Une souche d'Azotobacter du type 1, isolée de la terre C1 a été cultivée sur gélose-sels à l'ortho et au para-monoxybenzoate.

Cette même souche a été cultivée à l'état de pureté, dans 50 cc. d'une solution nutritive renferment, outre les élèments minéraux, 2,5 gr. pour 1000 de salicylate de sodium. A l'étuve à 30° C. la totalité de l'acide salicylique du milieu a disparu en 20 jours et on a noté une fixation d'azote correspondant à 7 mg. pour 1 gr. d'acide salicylique disparu.

Ensemencée sur plaque de silicogel de 200 mm. cette souche y faisait disparaître à la même température 0,5 gr. de salicylate en 4 jours. Le rendement de la fixation d'azote rapporté à la matière énergétique consommée (acide salicylique) était alors de 0,75 pour 100, chiffre voisin de la valeur 1 pour 100 souvent trouvée pour les cultures d'Azotobacter sur plaques mannitées.

Avec une souche du type 5, isolée de la terre J1, nous avons obtenu en la cultivant à l'état de pureté sur milieu électif liquide à 0,5 gr. pour 1000 de phénol une fixation de 9 à 11 mg. d'azote pour 1 gr. de phénol consommé.

RÉACTION DES AZOTOBACTER DU SOL Á UN APPORT DE COMPOSÉS PHÉNOLIQUES DANS LE MILIEU NATUREL

Les techniques de la méthode directe (Winogradsky) nous ont permis d'étudier les réactions biologiques de nos terres types à des apports de composés phénoliques.

Les examens microscopiques nous ont permis de discerner dans les terres J1 et C1 après apport de salicylate, un certain accroissement du nombre et de l'importance des colonies de Coccus moyens (diamètre de 1μ à 1,5μ) de la florule autochtone. Mais les résultats obtenus dans cette voie étaient loin d'avoir toute la netteté désirable.

Par contre nous avons obtenu de belles colonies d'Azotobacter sur plaques de terres (J1 et C1) moulées, après humidification convenable, et incorporation de 0,015 à 0,033 pour 100 de phénol, ou 0,1 à 0,2 pour 100 d'acides ortho ou para-monoxybenzoïques.

Le tableau 5 indique le nombre de colonies d'Azotobacter comptées sur plaques de 9 cm. de diamètre, après incubation en chambre humide à 30°.

TABLEAU 5

Matière énergétique pour	Phénol	Acides monoxybenzoïques	
		Ortho	Para
100 de terre.....	0,015	0,200	0,200
Nombre de colonies J1...	..	120	320
Nombre de colonies C1..	36	40	..

CONCLUSIONS

Nos recherches établissent à la fois que les Azotobacter peuvent attaquer les composés phénoliques et que le carbone des noyaux benzéniques intervient dans l'énergétique des fixations d'azote.

Elles mettent en évidence une notion nouvelle: l'attaque des acides monoxybenzoïques est avant tout conditionnée par la structure moléculaire de ces acides. Les préférences des Azotobacter pour telle ou telle substance alimentaire se manifestent jusque dans la structure intime des molécules.

Nous avons enfin classé les Azotobacter des terres examinées en un certain nombre de types physiologiques qui se distinguent par leur comportement respectif sur 5 milieux électifs de référence: silicogel au mannitol, au benzoate, au phénol, au para et à l'ortho-monoxybenzoate.

THE NATURE AND AMOUNT OF EXTRACELLULAR NITROGEN IN AZOTOBACTER CULTURES

C. KENNETH HORNER and DEAN BURK

Bureau of Chemistry and Soils, U. S. Department of Agriculture, Washington, D. C., U. S. A.

THE study of the formation of extracellular nitrogen in ordinary cultures of the free-living, nitrogen-fixing bacterium Azotobacter has received comparatively little attention. Several investigations have been conducted with a view to identifying intermediates in the nitrogen fixation process, though few have given sufficient recognition to criteria necessary for establishing a reasonably specific relationship between the occurrence of a particular compound and its role in nitrogen fixation as distinguished from life processes generally. It is very desirable from the practical or agrobiological standpoint to know to what extent the organic nitrogenous matter of Azotobacter cells is decomposed and liberated into the surrounding medium, both during the growth and during the autolytic phases of pure cultures of the organism.

The present report briefly summarizes findings obtained in an extensive systematic investigation, to be described in detail elsewhere, concerning the amounts, proportions, and kinds of soluble cell-free nitrogen compounds found in many hundreds of young and old Azotobacter cultures, in relation to common culturing variables, and with particular attention to possible differences between cultures grown in free and in fixed nitrogen. The excellent synthetic media now available for obtaining large quantities of Azotobacter nitrogen (20 to 50 mgm./100 cc.) make it possible to determine with much less qualitative ambiguity and with far greater quantitative precision than heretofore the kinds and amounts of extracellular nitrogen formed. The cultures used have been grown in thin layers in stationary flasks, or in continuously aerated gas wash bottles with due regard for maintenance of culture purity. A most important criterion of purity invariably employed, in addition to microscopic and other criteria, was virtual absence of growth upon inoculation of cultures to be tested into sugar-free, peptone-meat extract-asparagine media. Some twenty strains of *Azotobacter*, of the *chroococcum*, *vinelandii*, *agilis*, and *agilis* var. *atypica* types, were employed.

AMOUNT AND PROPORTION OF EXTRACELLULAR NITROGEN

Ten to twenty-five per cent of the total nitrogen in young Azotobacter cultures 1 to 4 days old was normally found in a soluble, noncellular form. Under good growing conditions, with adequate sugar, iron, molybdenum, and customary mineral salts, a total of 5 to 25 mgm. N/100 cc. medium was fixed during this period, of which 0.5 to 5 mgm. N/100 cc. were extracellular.

Various factors influence the quantities of soluble nitrogen obtained, one of the most important being age of culture. The amount and the proportion of nitrogen liberated by Azotobacter cells definitely increased with age and growth of culture. Even in the youngest cultures, the extracellular nitrogen was always at least 8 to 10 per cent of the total nitrogen.

Fifty to seventy per cent of the total nitrogen was usually the final maximum amount of soluble nitrogen found in cultures 1 to 3 weeks old in which the sugar or other available energy supply had disappeared, but occasionally values as high as 80 per cent were observed. With 1½ to 2½ per cent sugar initially, the total nitrogen ranged from 20 to 35 mgm. N/100 cc., but with still greater amounts of sugar it was possible to obtain 50 mgm. N/100 cc. A large proportion of the soluble nitrogen in old cultures unquestionably arises from autolysis of the cells, but it is our opinion that most of the 10 to 20 per cent soluble nitrogen observed in young cultures should be regarded as normal vital secretion.

Virtually all of the excreted nitrogen is organic, and constituted about 10 ±3 per cent of the organic carbon compounds liberated; this percentage is only slightly less than the nitrogen percentage most frequently observed in the cell material itself, and indicates considerable similarity between the extracellular and the cellular nitrogen.

TYPES OF SOLUBLE NITROGEN LIBERATED

Precipitable-N. The nitrogen present in supernatant, cell-free liquid from centrifuged cultures of Azotobacter has been differentiated in regard to precipitation by trichloracetic acid, phosphotungstic acid, aluminum sulfate, and lead acetate, as well as by dialysis through cellophane bags, as follows:

Age of culture	Average values of per cent soluble nitrogen precipitated by			
	Trichloracetic acid	$Al_2(SO_4)_3$	Phosphotungstic acid	Lead acetate
days				
2– 5	14	20	29	64
10–13	30	20	39	66
24	40	36	54	76

As might be expected, the trichloracetic acid-precipitable nitrogen, corresponding to protein nitrogen, showed the greatest relative variation with age; from a small proportion of the soluble nitrogen in young cultures (in individual cases less than 5 per cent), it approached half the total soluble nitrogen in older cultures where autolysis was well advanced. The phosphotungstic acid-insoluble fraction also increased with age of culture, but to a smaller degree; in young cultures it constituted over twice the trichloracetic acid-precipitable fraction, but in older cultures it was perhaps only a third greater. Alkaline aeration of the supernatants for some hours converted all of the trichloracetic acid fraction into a form no longer precipitable by this reagent but now precipitable by phosphotungstic acid; this was true not only for Azotobacter soluble protein but also for ordinary protein, like egg albumin. The nitrogen rendered insoluble by lead acetate was two-thirds to three-fourths of the total soluble nitrogen in virtually all cultures tested at any age, the proportion changing relatively little with age.

The results on precipitable-N presented here indicate that in ordinary Azotobacter cultures as grown by most investigators, a heterogeneous mixture of nitrogen compounds, ranging from simple substances to complex proteinaceous material, is liberated from the cells. The results concur in general, where they are comparable, with those obtained by J. G. Lipman with *A. vinelandii* at the turn of the century.[3]

Dialyzable-N. The soluble nitrogen in a young 2-day culture was about 75 per cent dialyzable through cellophane that allowed the passage of nitrogen compounds below a molecular weight of 6,000 to 10,000. In old cultures containing much more protein the dialyzable nitrogen, though increased in amount, was somewhat less in proportion, about 50 to 60 per cent. In several experiments Azotobacter was grown in cellophane bags surrounded by 6 to 12 times the volume of medium that was inside. During the period 1 to 9 days, successive analyses showed that more than 50 per cent of the total soluble nitrogen was obtained in the external solution, confirming the order of result just mentioned. The rates of growth and nitrogen fixation and the total amount of cellular nitrogen inside the bag were unaffected by the continuous removal of the extracellular nitrogen to the external solution around the bag.

Amino acid-N. Routine tests were made for amino acid-N, ammonia-N, hydroxylamine-N, oxime-N, and nitrite-N on most cultures involved in this investigation. Amino acid-N was ordinarily detectable experimentally (van Slyke gasometric or Folin colorimetric methods) in all cultures of Azotobacter containing 5 or more mgm. of total nitrogen utilized (from free or fixed nitrogen) per 100 cc. This type of nitrogen increased from 0.05 to 0.1 mgm. amino acid-N/100 cc. in young cultures to 1 to 2 mgm./100 cc. in older cultures. The percentage of amino acid-N, however, decreased with age,

from 10 to 40 per cent in young (1-4 day) cultures to 5 to 15 per cent in older cultures (10-40 days). This is to be expected from the observations mentioned earlier concerning the increase in proportion of more complex nitrogenous substances in the older cultures.

Virtanen[4] has reported that almost all the amino-N excreted by vigorous nitrogen-fixing Azotobacter cultures (as well as legume nodules) is aspartic acid (or, with legumes, partly beta-alanine), and has proposed a scheme involving this amino acid specifically in the nitrogen fixation mechanism. A preliminary determination of dicarboxylic acid amino-N in one of our older Azotobacter cultures, by the Foreman method, indicated a considerable fraction of the amino-N in this form, but the method and quantities of soluble-N available permitted no more than semiquantitative observation. As has already been indicated, however, the amino acid-N is not a large or predominant part of the soluble-N; it is only one of a number of heterogeneous soluble nitrogen compounds produced, and it occurs, moreover, to an equal or greater extent in cultures grown on fixed nitrogen compounds (nitrate, ammonia, urea) instead of free nitrogen. The *specificity* of aspartic acid as an intermediate in the fixation mechanism is, therefore, of questionable significance, as far as Azotobacter is concerned.

Ammonia-N. Ammonia, as we have upon various previous occasions indicated, is rarely if ever detected in *free* nitrogen cultures until the energy supply is very nearly depleted. In our earlier work on the ammonification of autolyzing cultures,[2] in which stock aeration-flask cultures were centrifuged, washed, and incubated in concentrated suspension under not strictly sterile or pure culture conditions, a maximum of about 50 per cent of the cell-N was obtained as NH_3-N under aerobic conditions and in the necessary absence of energy material, after some hundred hours' incubation. We have now repeated this work under strictly pure culture conditions maintained throughout every operation, including centrifugation, washing, and incubation, and find that *pure* cultures of Azotobacter yield a lower maximum of but some 8 to 12 per cent NH_3-N. This value is essentially of the same order as (or only slightly greater than) the maximum yields obtained by Roberg, Winogradsky, and Kostyschev in recent years. The additional odd 40 per cent obtained by us in our earlier published work is to be definitely ascribed, we now believe, to the influence of contamination developing rapidly during incubation. This we could easily demonstrate in the new experiments being reported, for upon the addition, to the pure cultures, of certain contaminants obtained from cultures which had been deliberately exposed to and contaminated by air, the yield of NH_3-N was increased from the recent value of about 10 per cent up to the old value of 50 per cent. Not all contaminants increased the yield, but those which were effective appeared to act upon every strain of Azotobacter tested, even though, as we have now found,

some strains yield by themselves in pure culture almost no NH_3-N. As we have now ascertained, in working with over a dozen strains, the pure cultures yielding the most ammonia (up to 12 per cent of the total nitrogen) are apparently of the *A. vinelandii*, green-fluorescing (high N-content) type, and those yielding least NH_3-N are of the intense black, *A. chroococcum* (often gummy, low N-content) type, the dark or light brown *A. chroococcum* types being in general intermediate. A more recent careful study of the microscopic appearance of Azotobacter cells undergoing ammonification shows little change in pure cultures which yield the 12 per cent NH_3-N of total-N, but (contrary to previous indication) a major disintegration with contaminants present yielding 50 per cent NH_3-N of total-N.

Our earlier work, therefore, must now be regarded, as rightly inferred in most, though not all, particulars by Winogradsky in his critical memoir,[5] as involving contaminants in part. The extent to which contaminants influenced the reported ammonification as a function of anaerobiosis, pH, temperature, toluene, cyanide, and added fixed nitrogen substrates capable of being ammonified like Azotobacter-N, remains to be restudied. The reported recovery after pasteurization was undoubtedly due to subsequent contaminant development under the not strictly sterile conditions then maintained.

On the other hand, we have now definitely ascertained that with the pure cultures also, the *main* condition for aerobic ammonia production is *absence of oxidizable matter*, such as sugar, which absence permits autolysis to set in rapidly. Though our previously published conclusion as to the maximum NH_3-N produced spontaneously by Azotobacter must, therefore, be quantitatively modified, our general conclusions pertaining to the agronomic importance of the decomposition of Azotobacter cell nitrogen (greatly reinforced in the present paper), and the significance of the observed NH_3-N in the mechanism of the fixation process, are still valid. As was concluded earlier, the extracellular ammonia observed heretofore, in our own and in all previous investigations by others, has been derived entirely from the decomposition of normal Azotobacter nitrogen upon depletion of readily available organic matter from the medium, and not, in any measurable quantity, by direct synthesis from free N_2; the ammonia observed has been liberated *after*, not *before*, a synthesis into cell nitrogen, and the occurrence of any ammonia as an essential intermediate in the specific mechanism of nitrogen fixation, although possible, still remains to be demonstrated. In this connection our previously advanced extensive critique of results obtained hitherto in the field as a whole (2, p. 83, 113-117) remains as pertinent and applicable at the present time as when written: so far as NH_3 is concerned in nitrogen fixation by Azotobacter, "The mystery of the intermediate catalytic processes is *more alive* than ever."

Cultures given 200-400 p.p.m. nitrate as a source of nitrogen will, in many instances, be found to contain up to 10-20 p.p.m. NH_3-N. The same quantities may be found with asparagine, and considerably more will be given by urea if for some reason growth is somewhat slowed up. The urea will then be largely hydrolyzed to NH_3, whereas under more favorable conditions the NH_3 does not appear, presumably because it is consumed as rapidly as it is produced.

Nitrite-N. Nitrite is detected only in nitrate cultures, usually at a concentration of 1 p.p.m. or much less, but occasionally 10-15 p.p.m. will be observed. Here again, the rate of growth markedly influences the amount found. Whether all nitrate or only some is converted to nitrite first, on its way to being converted into cell nitrogen, is an open question.

NH_2OH-N and Oxime-N. Using the Endres-Blom tests on many hundreds of cultures over a period of several years, we have observed NH_2OH only occasionally in cultures of *A. vinelandii*, out of some twenty strains tested, and always at a concentration of less than 0.05 p.p.m., and generally less than 0.01 p.p.m. We have never detected oxime-N in *A. vinelandii*, but have done so rather regularly in three strains of *A. chroococcum* and occasionally in three other strains of this species, but not as yet in any of some dozen other miscellaneous strains. When oxime-N is detected, the concentration is of the order of 0.01-0.1 p.p.m. (in terms of nitrite-N formed in the test), and occasionally one strain is shown 1-2 p.p.m., which is much higher than Endres has reported, in terms of nitrite-N.[1] These values, in terms of nitrite-N, are undoubtedly low, but as we shall show elsewhere, the Endres correction factor of 6-fold is almost always arbitrary, and, depending upon conditions, may easily vary from unity to over 10.

INFLUENCE OF CULTURING VARIABLES

The important factor of age of culture as an influence on the quantities of total soluble nitrogen excreted as well as on the gross fractionation and proportion of amino acid nitrogen has already been brought out. A number of variables, such as aeration, nature and concentration of energy source, individual inorganic constitutents of the medium, stimulants such as agar, and strain, were not observed to produce any very notably different picture in regard to the quantitatively significant extracellular nitrogen, except, as indicated, for different strains in regard to production of NH_2OH, NH_3, and oxime. Molybdenum commonly increased very considerably the amount and proportion of soluble nitrogen, but this was almost certainly an indirect effect and due to the greatly increased growth. In general, the greater the growth the greater were both the amount *and* proportion of soluble nitrogen, and in this sense the aforenamed factors played a role.

INFLUENCE OF ADDED FIXED NITROGEN

Next to age of culture, probably the most important factor to be considered in regard to excreted nitrogen, and certainly the most important with respect to intermediates in the nitrogen-fixing mechanism, is the effect of added fixed nitrogen. The total extracellular nitrogen other than that added, that is, that which is transformed, is in general somewhat greater in cultures given nitrate, ammonia, urea, or asparagine than in those given only free nitrogen. It is commonly two to three times as great in young comparable cultures. Again, the difference is dependent somewhat on the extent of growth; but apart from this, it seems slightly easier for Azotobacter to give off nitrogen of soluble organic nature from fixed than from free nutrient nitrogen, especially from ammonia. Nevertheless, the difference is only mildly quantitative, and no qualitative differences have been observed as yet, except for the obvious cases of nitrite from nitrate, and ammonia from nitrate, urea, or asparagine in the presence of adequate sugar (nonautolyzing conditions); the NH_2OH-N, oxime-N, amino-N, cytochrome-N, flavin-N, and various precipitable and dialyzable nitrogen fractions appear with both free and fixed nitrogen, and, so far as observed, in not very greatly different proportions of the total nitrogen transformed, more, if anything, being obtained with fixed nitrogen.

CONCLUSION

From the agronomic point of view, this paper indicates that a considerable part, one-quarter to one-half, of the nitrogen which might be fixed by Azotobacter in soils, or of the inorganic fixed nitrogen that might be utilized, can be liberated in a soluble extracellular organic form, spontaneously without the necessary intervention of other microbiological agents. Pure cultures of Azotobacter can convert up to about 10 per cent of their total nitrogen into extracellular ammonia nitrogen; but for the conversion of virtually all the extracellular organic nitrogen liberated by Azotobacter into ammonia, other organisms are required.

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STUDIES ON AZOTOBACTER AND NITRIFYING ORGANISMS IN RELATION TO VARIOUS FERTILIZER TREATMENTS IN SOILS UNDER CITRUS CULTURE CONDITIONS

M. WINNIK and MIRIAM GOLDBERG

Mikveh-Israel Experimental Station, Palestine

THE work here presented forms a part of microbiological studies carried out on soils taken from citrus orchards. These soils, mostly red loamy sands, are naturally poor in organic matter and deficient in the ordinary plant nutrients; when under citrus culture, they require irrigation and a continuous supply of nitrogen. Various sources of nitrogen, organic and mineral, are used, with or without added phosphorus and potassium. It appears that the activity of such aerobic bacterial processes as nonsymbiotic nitrogen fixation and nitrification, under the favorable conditions of moisture, temperature, and aeration that prevail in these soils, may be determined to a high degree by the kind and amount of fertilizers used. More definite information regarding the effect of manuring on the two aforementioned bacterial processes seems to be of importance both theoretically and practically. An attempt was made to investigate the activity of Azotobacter and of nitrifying organisms in its relation to various forms of mineral nitrogen (sulfate of ammonia, nitrate of lime, and nitrate of soda) used alone or with added phosphorus and potassium and green manure.

In these investigations use was made of the soils from our lysimeter experiments with citrus trees. The many and varied factors which come into play under natural orchard conditions are carefully controlled in the lysimeters. The experiment was started on 10 lysimeters in 1926, for the study of citrus fertilization problems. Each lysimeter is a large cylindrical tank 1.2 m. deep with a surface area of about 2 sq. m. All tanks were filled with the same kind of soil, a red loamy sand, typical of soils used for citrus culture. A young budded orange tree planted in each lysimeter in the spring of 1926 has since developed into a fruit-bearing tree, from which nine crops have been harvested.

During the first 3 years of the experiment lupine was grown as green manure in all lysimeters, except two, left as controls. In addition to green manure, a dressing of double superphosphate and sulfate of potash was given

to six of the remaining lysimeters. Since the end of the 3-year period, green manure has been continued in four lysimeters only, the green manure being grown in the lysimeters and elsewhere. To each of these four, during the last 9 years of the experiment, was added a total of 95 kgm. of green organic matter, which is equivalent to an addition of about 300 gm. of nitrogen. The application of mineral nitrogen in various forms started at the beginning of the fourth year of the experiment. This differentiation in treatment was maintained throughout the following 9 years. The actual total amount of nitrogen received by each tree, except those in the control lysimeters, during the 9 years is the same, about 1 kgm., the only difference in treatment being that the source of the nitrogen varied.

The treatment of each lysimeter is shown in table 1.

METHODS

The methods of Winogradsky[1, 2, 3] were employed as follows:

1. Plate cultures on elective silica-gel. Petri dishes 9 cm. and 20 cm. in diameter were used. The counts of colonies developed from a definite weight of soil, sprinkled on the plate surface, were made after 48 and 72 hours of incubation for the Azotobacter test, and after 10, 14, and 21 days for the nitrification test. All numbers are calculated per gm. of soil. No less than two plates were used for the examination of each soil sample.

2. When the large silica-gel plates were used, the corresponding determinations of the gain of nitrogen, or the amount of nitrites formed, were made after 10 and 15 days of incubation respectively.

3. Kneaded earth plates for the determination of Azotobacter activity. This method served also for determining the phosphorus deficiency in all samples of soils.

Methods 1 and 3 were used chiefly because of their easy technic, coupled with their sensitiveness in the differentiation of various soils. The silica-gel plate method, recently worked out by S. and H. Winogradsky[3] for studies on nitrifying organisms was used most extensively by us.

The samples for microbiological studies were taken periodically at various seasons beginning in 1934, and the results were compared with all changes, both chemical and physicochemical, especially in the availability of nutrients, which took place in the soils. Samples were taken at depths of 0-20 cm., 20-40 cm., and 40-60 cm., each sample being a composite of four borings. The soils were mixed, air-dried, and allowed to stand for 24 hours before examination.

RESULTS

The recorded counts of colonies on silica-gel plates and the degree of development of Azotobacter on soil plaques are the averages of many deter-

minations carried out with samples taken at different periods of the year from 1934 to 1938. Although the absolute numbers of bacteria fluctuate in the different seasons, the general trend of bacterial activity of the soil under each treatment remains unchanged and has its own characteristics. It appears to be justifiable, therefore, to omit the discussion in detail of all data obtained and to present only the averages.

Azotobacter activity. The comparative investigations of the soils from the lysimeters which received fertilizers and from those left as controls lead to a conclusion similar to that of Winogradsky[2] and Ziemiecka,[4] that the addition of nitrogenous fertilizers to the soil may effect a considerable reduction in Azotobacter activity. It may be noted from the results in table 1 that the density of Azotobacter cells is the greatest in the soils of the lysimeters which received no fertilizers at all (Nos. 1 and 6).

The injurious effect of nitrogen is not of the same degree in differently treated soils. It is most pronounced in the soils of those lysimeters which received only nitrogen without the addition of phosphorus and potassium (Nos. 2 and 2A); in other words, in soils highly deficient in these two nutritive elements.

Subsequently, the injurious effect of nitrogen is less pronounced in the soils which received minerals in addition to the nitrate nitrogen (Nos. 3 and 5), and it is only slightly marked in those soils which were provided with a large quantity of green organic matter in addition to the aforementioned fertilizers (Nos. 3A and 5A).

It is to be regretted that our lysimeter experiments lack a treatment in which minerals alone are added to the soil. This treatment has been omitted since it is of no practical importance in citrus culture. It may be supposed that the number of Azotobacter in control soils is reduced by the lack of available phosphate. The difference between the degree of Azotobacter activity in the soil treated with minerals alone, as compared with those treated with nitrogen could be expected to be more pronounced.

In the soils treated with sulfate of ammonia (Nos. 4 and 4A) the activity of Azotobacter is low, although these soils were provided with a sufficient amount of available phosphorus, potassium, and green organic matter. It may be suggested that this injurious effect is connected with some chemical and physicochemical changes produced by the continuous use of sulfate of ammonia. Indeed, the periodical observations carried out in these soils have shown a reduction in calcium carbonate content, buffer and base exchange capacity, as compared with the soils under other treatments. There is also a marked fluctuation in the pH value of these soils, which drops from 7.4 to 6.7 about a month after the application of sulfate of ammonia and its almost entire transformation into nitrates. This case is an interesting example that in soil investigation the bacterial factor may serve as an index of

TABLE 1

AZOTOBACTER ACTIVITY

Lysimeter number	Depth*	Treatment†	Silica-Gel plates		Kneaded earth plates	
			Count per gram of soil on petri dishes 9 cm. in diameter	Nitrogen fixed per 2 gm. of mannite on petri dishes 20 cm. in diameter	Growth on control plate	Growth on plate with phosphate
				mgm.		
1.....	1	Check	800	20	0	++++
	2		600			
	3		150			
6.....	1	Check	1,000	20	0	++++
	2		760			
	3		120			
2.....	1	N as $Ca(NO_3)_2$	75	12	0	+
	2		30			
	3		0			
3.....	1	N as $Ca(NO_3)_2$ + P + K	300	17	traces	++
	2		120			
	3		30			
4.....	1	N as $(NH_4)_2SO_4$ + P + K	100	13	+	+
	2		100			
	3		170			
5.....	1	N as $NaNO_3$ + P + K	460	20	++	+++
	2		240			
	3		170			
2A.....	1	N as $Ca(NO_3)_2$ + G.M.	190	14	0	+
	2		90			
	3		60			
3A.....	1	N as $Ca(NO_3)_2$ + G.M. + P + K	700	19	++	+++
	2		680			
	3		180			
4A.....	1	N as $(NH_4)_2SO_4$ + G.M. + P + K	30	8.8	+	+
	2		90			
	3		180			
5A.....	1	N as $NaNO_3$ + G.M. + P + K	650	20	++	+++
	2		500			
	3		180			

* 1 = 0–20 cm., 2 = 20–40 cm., 3 = 40–60 cm.

† K as sulfate of potash, P as double superphosphate, G.M. = green manure.

some physicochemical changes in the soil. It is worthwhile to note that the fertility of the soil from different lysimeters, as measured both by the development of the trees and by the size of crops, does not correlate with Azotobacter activity.

The difference between the effect of two forms of nitrate nitrogen—$NaNO_3$ and $Ca(NO_3)_2$—on the activity of Azotobacter is pointed out in the data relating to the soils of lysimeters 3 and 5, treated not only with the aforementioned forms of nitrogen, but also with minerals, but without the application of green organic matter.

It may be suggested, that the more favorable effect of $NaNO_3$ as compared with that of $Ca(NO_3)_2$ can be explained by the different contents of available mineral nutrient, especially phosphorus in soils. By the use of $Ca(NO_3)_2$ the availability of phosphorus has been reduced, as shown in table 2. The activity of Azotobacter is reduced in the deeper layers of soil. The differences in bacterial activity due to the different treatments also are pronounced at the depth of 20-40 cm. and are slightly noticeable even at the depth of 40-60 cm.

In the soils treated with sulfate of ammonia the number of Azotobacter colonies, on the contrary, slightly increases with depth. This is due perhaps to the increased amounts of $CaCO_3$ in these layers.

TABLE 2

DISTRIBUTION OF WATER-SOLUBLE PHOSPHATE IN LYSIMETER SOILS AT DIFFERENT DEPTHS

(Averages of several determinations made in samples taken at the period of the lowest content of phosphate in soils)

Lysimeter number	P_2O_5 per kgm. soil			Lysimeter number	P_2O_5 per kgm. soil		
	0–20 cm.	20–40 cm.	40–60 cm.		0–20 cm.	20–40 cm.	40–60 cm.
	mgm.	*mgm.*	*mgm.*		*mgm.*	*mgm.*	*mgm.*
1.....	0.6	0.5	0.4	6.....	0.5	0.5	0.4
2.....	0.5	0.4	0.4	2A.....	0.8	0.6	0.5
3.....	4.6	2.3	2.0	3A.....	7.4	6.2	4.0
4.....	8.1	7.8	7.1	4A.....	8.1	8.1	5.1
5.....	8.8	7.3	7.2	5A.....	8.5	7.0	5.3

The activity of nitrifying organisms. There is an extensive literature on the relation of various fertilizers to the nitrification process in soil. In almost all investigations the nitrifying power was determined by adding nitrogenous fertilizers to the soil and measuring the amount of nitrates formed under field or laboratory conditions. In but few investigations has the

density of nitrifying organisms, as measured by the plate method, been taken as an index of soil nitrifying power. Without reviewing the literature, we shall mention only that most investigators who used for this purpose either the first or the second method have reported that the application of nitrogenous fertilizers increases the nitrifying power of the soil. As to the effect of minerals, no definite results have been obtained. In some investigations no relationship between these two factors was observed, whereas in others the application of minerals caused an increase in nitrifying power.

The data given in table 3 show that the application of nitrogen, minerals, and green manure, each in its way, stimulates the nitrifying power of these soils, poor in nutrients.

The number of colonies in the control soils (Nos. 1 and 6) is very low. It is increased to a slight degree by the application of nitrate nitrogen alone, and to a much greater degree by the addition of minerals and nitrogen, as may be seen by comparing the data from lysimeters 2 and 3.

The most stimulating effect is produced by the application of green organic matter (Nos. 2A, 3A, 4A and 5A). Nitrogen as sulfate of ammonia increases both the numbers and the rapidity of development of the nitrifying organisms to a much greater degree than do the two nitrate forms of nitrogen. This prevailing effect of sulfate of ammonia is especially marked in the data from deeper layers as compared with data from corresponding layers of other lysimeters. It may be noted that the differences in the numbers of colonies resulting from various soil treatments are in some cases more pronounced in the deeper layers. The presence of available mineral nutrients in these soils appears to be one of the limiting factors for the activity of nitrifying organisms.

The differences in the numbers of colonies between the soils treated with various forms of nitrate nitrogen were in favor of the soil treated with $NaNO_3$. As in the case of Azotobacter, the difference is most marked in those soils which did not receive green manure, and it is probably due to the greater amount of available phosphorus.

The results of the work with the lysimeter soils are in agreement with the data obtained from our extensive investigations on various soils taken from different citrus groves, some typical data from which are shown in table 4. The activity of nitrifying organisms in these soils positively correlates with their nitrogen, organic matter, and mineral contents. The most active soils are those which have the highest nutrient content. The injurious effect of nitrogen fertilizers on Azotobacter takes place mostly in soils with a low phosphorus content. In some soils of a relatively high nitrogen content but rich in phosphorus, the presence of Azotobacter in an active state could be ascertained.

The aforementioned conclusions concerning the bacterial activity of various

TABLE 3

ACTIVITY OF NITRIFYING ORGANISMS

Lysimeter number	Depth*	Count per gram of soil			Nitrites† formed on large silica-gel plate per gram of soil
		After 10 days	After 14 days	After 21 days	
					cc.
1	1	0	60	600	1.6
	2	0	0	45	
	3	....	....	....	
6	1	0	80	540	1.5
	2	0	0	60	
	3	....	....	....	
3	1	165	733	3,000	33.6
	2	0	45	535	
	3	....	....	....	
4	1	560	2,525	3,750	63
	2	270	1,530	3,000	
	3	0	400	....	
5	1	272	1,240	2,800	46
	2	0	300	1,040	
	3	0	150	....	
2A	1	1,025	3,600	....	64
	2	0	300	435	
	3	0	30	....	
3A	1	1,220	3,200	....	65
	2	30	600	1,350	
	3	0	60	....	
4A	1	1,340	2,800	....	63
	2	540	2,000	4,000	
	3	0	435	....	
5A	1	900	3,000	....	70
	2	180	800	1,465	
	3	0	165	....	

* 1 = 0–20 cm., 2 = 20–40 cm., 3 = 40–60 cm.
† Measured by amount of 0.1 *N* $KMnO_4$ used for titration.

citrus soils are valid only if the pH factor is constant, as nitrifying bacteria, and especially Azotobacter, in these soils which have a low buffer capacity, are very sensitive to changes in the pH value; in fact, their activity is greatly reduced at pH 6.6-6.7.

TABLE 4

ACTIVITY OF AZOTOBACTER AND NITRIFYING BACTERIA IN VARIOUS CITRUS ORCHARD SOILS IN RELATION TO pH, TOTAL NITROGEN, AND WATER-SOLUBLE PHOSPHATE CONTENT OF THESE SOILS

Soil number	Age of grove	pH	Nitrogen	Water-soluble P_2O_5 per kgm. soil	*Azotobacter* activity				Nitrifying organisms per gram of soil after 14 days
					Count per gram of soil	Growth on the kneaded earth plates		Nitrogen fixed per 2 gm. mannite	
						Control	With P		
	years		*per cent*	*mgm.*				*mgm.*	
193	25	7.7	0.13	30	90	+	+	19.8	4,300
83	25	7.7	0.105	30	600	+++	+++	19.8	2,800
42	25	7.2	0.11	30	700	++++	++++	20.0	5,400
9	25	7.3	0.13	20	660	++++	++++	19.0	3,000
181	25	7.8	0.10	20	240	++	++	18.5	3,400
177	25	7.4	0.084	14	210	++	++	....	2,670
435	25	7.6	0.067	6	450	+	+++	....	1,230
17	25	7.9	0.083	20	750	++++	++++	19.0	2,500
109	25	7.4	0.035	7	100	+	++	15.0	465
27	25	7.0	0.056	5	250	0	++	12.0	1,000
357	9	7.6	0.035	1.0	45	0	+	15.2	300
105	7	7.2	0.044	20	210	+++	+++	17.2	1,050
229	5	7.7	0.044	trace	90	0	+	....	180
161	5	7.2	0.066	2	75	0	+	12.0	210
441	5	7.0	0.038	2	60	0	+	....	820
265	5	7.4	0.03	trace	20	0	+	....	465
417	5	6.6	0.042	trace	0	0	0	1.5	0
413	5	6.6	0.03	trace	0	0	0	....	30

SUMMARY

The purpose of this work was to study the activities of Azotobacter and nitrifying organisms in soils under various treatments including comparisons of various forms of mineral nitrogen [$Ca(NO_3)_2$, $NaNO_3$, $(NH_4)_2SO_4$] with and without the addition of P, K, and green manure.

The investigations were performed on soils from carefully controlled lysimeter experiments with citrus trees.

The Winogradsky methods were used for bacterial activity determinations.

The results may be summarized as follows:

Azotobacter. The addition of nitrogenous fertilizers, in general, reduces Azotobacter activity, and the extent of its injurious effect depends on the form of mineral nitrogen used and on the presence of available minerals, especially of phosphorus and of green organic matter. In the two soils

treated with nitrate nitrogen in addition to P, K, and green manure, the density of Azotobacter growth is almost as great as that in unfertilized soils. The number of Azotobacter colonies diminishes in soils similarly treated but without addition of green manure, and is reduced to the greatest extent in soils treated with nitrogen alone, or with nitrogen and green manure but without the addition of P, K. Nitrogen applied as sulfate of ammonia has a markedly injurious effect even in the presence of P, K, and green manure.

Nitrifying organisms. The three factors—nitrogen, green organic matter, and minerals—investigated in this work in relation to bacterial activity, each produce a stimulating effect on the activity of nitrifying organisms. Nitrogen as sulfate of ammonia increases the development of nitrifying bacteria more than does nitrogen as $NaNO_3$ and as $Ca(NO_3)_2$. The stimulating effect of available minerals on nitrifying organisms is clearly marked in soils which did not receive green manure. This effect is also marked in the second layers of all soils.

The results of an extensive investigation of various citrus grove soils are briefly mentioned, and their similarity to the results obtained in this work is emphasized.

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LIST OF PAPERS ON SOIL MICROBIOLOGY PRESENTED BEFORE THE THIRD INTERNATIONAL CONGRESS FOR MICROBIOLOGY IN NEW YORK CITY

THE SOIL POPULATION

1. Dr. H. J. Conn, N. Y. Agr. Exp. Station, Geneva, N. Y., U. S. A.: "Characteristics and Relationships of the Autochthonous Flora of the Soil."
2. Dr. A. G. Lochhead, Central Experimental Farm, Ottawa, Canada: "The Qualitative Nature of the Bacterial Soil Flora with Special Relation to Productivity and the Rhizosphere."
3. Dr. R. L. Starkey, N. J. Agr. Exp. Station, New Brunswick, N. J. U. S. A.: "The Influence of Plants upon the Soil Population."
4. Dr. T. Gibson, Edinburgh and East of Scotland College of Agriculture, Edinburgh, Scotland: "Investigations of the Bacterial Flora Characteristic of Soils."
5. Dr. D. Fehér, Kgl. Ung. Palatin Josef Universität, Sopron, Hungary: "Die komplexe Wirkung der Bodentemperatur und der Bodenfeuchtigkeit als regulative Grundfactoren des Bodenlebens."
6. Dr. C. Stapp, Biolog. Reichsanst. f. Land. und Forstwirtschaft, Berlin-Dahlem, Germany: "Ein neues freilebendes stickstoffbindendes Bakterium."
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11. Dr. T. L. Martin, Brigham Young Univ., Provo, Utah, U. S. A.: "The Occurrence of Algae in Some Virgin Utah Soils."
12. Dr. J. E. Greaves, Utah Agr. College, Logan, Utah, U. S. A.: "Some Factors Influencing Nitrogen Fixation by *Azotobacter chroococcum*."

13. Dr. N. James, University of Manitoba, Winnipeg, Canada: "The Errors of the Plating Method."
14. Dr. S. F. Snieszko, Jagellonian Univ., Krakow, Poland: "Bacteriological and Biochemical Studies on a New Capsulated Bacillus."