A Study of the Fixation of Nitrogen by the Azotobacter.

A Thesis

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A Study of the Fixation of Nitrogen by the Azotobacter.

The problem of the Nitrogen supply has been one that has received a great deal of attention from the scientists. The chemists long ago knew that there was very little combined Nitrogen found in rocks, not nearly enough to supply the needs of plants. When it was found that the atmosphere was composed of a mixture that was four fifths nitrogen it was concluded that the problem was solved. Nothing could be easier than that the plants took their supply directly from the air. This idea was held for some time until it was demonstrated that the plants could not make use of nitrogen unless it was supplied in the combined form from the soil. A number of theories were soon advanced to account for the supply that was present in the soil.

Liebig brought forth the ammonia theory and this was accepted for some time. His theory was that ammonia was the important source of nitrogen for the plants. Liebig said that the natural supply of ammonia was sufficient for the plants and that in cases where the fertility of land had gone down it was caused by lack of other mineral ingredients. It was shown then that the amount of ammonia brought down to the soil by the rain and snow amounted to but a few pounds per acre. Liebig immediately maintained that the plants were capable of absorbing the ammonia from the air. It was finally established that the amount of ammonia in the air could not supply the amount of nitrogen needed by the green plants, and so the theory had to be discarded.

Between 1860 and 1870 it was generally believed that the nitrogen for plant food came only from the soil. It was known that plants

annually removed large quantities of nitrats from the soil and that drainage water carried away as much more and thus the source of all this nitrogen had to be found. The nitrate theory then advanced was that not only could ammonia and nitrates be formed from the decay of humus but that the oxygen and nitrogen in the soil could combine by chemical action and form nitrates.

In 1877 Schloesing and Muntz (1) first established the fact that nitrification was a bacteriological process. In this process however it was necessary for the bacteria to have nitrogenous organic matter to form these nitrates. Considering the small amount of nitric acid brought down by the rains and the great losses sustained by the soil; it was plainly seen that the great question as to how the combined nitrogen supply was maintained was still unsolved. In 1886 this was answered to a great extent by Hellriegel and Wilfarth's work (2) showing that some plants, the legumes, when aided by bacteria, could use the free nitrogen of the air. But it was known that fields allowed to run wild would increase in nitrogen content even without the presence of legumes. This then seemed to indicate that there was still another force acting.

Even before Wilfarth and Hellriegel's work on leguminous plants, Berthelot (3) had been carrying on some investigations as to the action of free nitrogen on uncropped soil. He took 50 Kg. of air dried soil kept it in a vessel exposed to rain and air for seven months; which after correcting for combined nitrogen brought down by rain, showed that the nitrogen content had increased 25%. The following are Berthelot's figures:

Nitrogen content at the beginning	50.37 gr.	Nitrogen content at close.	62.48 gr.
Added by rain / as NH_3	.0477"	Lost by drainage.	.674"
/as NO_3H	.0012"	" " " NH_3N	?
	50.42 gr.		63.15 gr.

In another experiment in which he had washed the soil free from nitrates he got an increase of 46%. In other soils he got varying amounts of fixation but in each case they showed a decided increase. He also found that when the soil had been steamed at 100 degrees centigrade that no increase of nitrogen could be obtained therefore he concluded that living organisms were responsible for the increase. He however, could not by plate methods isolate any organism that would assimilate free nitrogen.

Before the isolation of any nitrogen fixing bacteria the belief had gained ground that the algae could use free nitrogen. The reason for this belief was that the algae grew in places where there seemed to be no nitrates, such as on rockes and sand. In 1888 (4) Frank observed algae growing on the surface of pure sand and upon analysis found an increase in the upper layer as compared with sand on which no algae were growing. In 1892 (5) Schloesing and Laurent demonstrated that a soil in a closed vessel, if exposed to light and algae allowed to grow on it, would increase in nitrogen. They did not use sterile soil nor pure cultures of algae. Kossowitch(6)using pure cultures of algae did not get fixation but upon mixing with soil bacteria he obtained positive results. Later

Krüger and Schneidewing (7) got no fixation when they experimented with pure cultures of Chlorophyceae. These experiments showed that the algae could not of themselves assimilate free nitrogen but in connection with soil bacteria nitrogen was fixed. It is possible that the algae produce the carbohydrates which furnish the energy for the bacteria.

In 1894 Winogradsky (8) isolated a bacterium which he called Clostridium pasteurianum. This fixed nitrogen while growing as pure culture in nitrogen free solution containing dextrose. This organism is an anaerobe and is one of the butyric acid group of bacteria. In pure cultures for every gram of dextrose consumed two milligrams of nitrogen were fixed. Although the organism is an anaerobe it will grow in the presence of air provided other kinds of bacteria are present. This was found quite plentiful and widely distributed. But this organism was an anaerobe and it had often been observed that the fixation of nitrogen was strongest in soil that was well aereated so it seemed probable that a more powerful nitrogen fixer was still undiscovered.

In 1901 Beijerinck published a report on two new nitrogen fixers. He had found that when he inoculated media containing dextrose with earth that he would get the Clostridium Pastorianum, but that when he substituted mannit or Ca or Na propionate for the glucose that he got a heavy growth of a large organism that he called Azotobacter Chroococcum. He found this plentiful and widely distributed. He discovered these could be easily isolated and grown on mannite agar.

Beijerinck isolated two forms of this ; form one, the azotobacter chroococcum isolated from garden earth, but few individuals

of which were motile by reason of a single cilia. The second form, azotobacter agilis which he isolated from canal water at Delft; this was very motile having bundles of polar cilia. Beijerinck obtained fixation in impure cultures up to 7 mg. per gram. of mannite. In pure culture he got a very little fixation. In a second work with Van Delden (10) he changed his opinion as to the fixation powers of pure cultures of azotobacter. His conclusions in this latter publication were that the azotobacter could not fix nitrogen in pure culture, but that a symbiosis with other bacteria such as Granulobacters, Radiobacters and Aerobacter was necessary. His experiments in mixed cultures showed that with these he got fixation up to 5.9 mg. nitrogen per gram. mannite.

Even before Beijerinck's second paper had been published Vogel and Gerlach (11) had obtained good fixation of nitrogen on culture media inoculated with pure cultures of Azotobater Chroococcum. Later Freudenreich, (12) Koch (13) and Kröber established beyond doubt that the pure culture would fix free nitrogen.

Lipman (14) isolated three different azotobacter species of which the most powerful nitrogen fixer was an organism which he called azotobacter vinelandii. He found that although he got good fixations in pure culture, he got better fixation when the azotobacter was grown in symbiosis with another organism even though the second organism did not have the power to fix nitrogen by itself.

In late years Azotobater have been isolated from many different soils in practically every country. Different species have been found which fix nitrogen with varying degrees of effectiveness. One feature of their distribution has been that they are always found in abundance in soils having a vigorous growth of

Blue Green algae. Stoklasa(19) and Stranak, (26) both reported this phenomenon. Keutner (30) says that the azotobacter are widely distributed in the ocean occuring on algae and plankton organisms. He found that they had the power of fixing nitrogen in an eight per cent solution of salt. Keutner also found the organisms present in fresh water. Benecke (31) reported their presence in the Bay of Naples. Stoklasa said that the only soils in which he was unable to find them were in samples of the high lying soils of the Alps.

The fact that these bacteria might have a great deal to do with maintaining the fertility of the soil has caused much study to be made of their requirements. The Azotobacters are very sensitive to the amount of aeration. They are highly aerobic, and Krainskii(15) says that the amount of water in the soil will affect the development of the bacteria by the exclusion of the air by the moisture. He found that the fixation in large quantities of culture media was small on account of the lack of aeration. According to other investigators, no fixation of nitrogen in artificial cultures was found at temperatures below 5° C or above 50° C. Their optimum temperature is between 18° and 31° C.

Azotobacter are very sensitive to changes in their food supplies, in fact so much so that Christensen (16) has tried to work out a biological method for determining the available plant food in a soil by using azotobacter. He did this by inoculating the soils into culture media containing different amounts of plant food and then noticing the difference in development of the azotobacter.

In fixing nitrogen the azotobacter are dependent for energy on some fermentable carbohydrate. In artificial media sugars are generally used, as mannite seems to be the most efficient, it is most often used. Hoffman and Hammer (17) tested out a number of sugars. In impure culture they got the highest fixation from lactose while mannite was almost as good. In pure cultures they got fixation from practically all the sugars but the highest from mannite. In the pure culture the lactose was very much poorer as a source of energy. Heinze (18) found that the pectin and pentosan substances were not as efficient sources of energy as the sugar. Stoklasa (19) says that in the soil the furfuroids make the best food for the azotobacter. Krainskii (15) states that for every 1 part of nitrogen fixed 90 parts of C are used. There has been some discussion as to whether the azotobacter can use cellulose to furnish energy. Lipman (20) in experiments in which he added different amounts of filter paper to pure cultures found that up to a certain amount he got an increased fixation of nitrogen. But that when he added a large amount of filter paper, it seemed to inhibit the action. Warmbold (32) found that humus favored fixation. It has been noted by others that soils containing humus are stronger in fixing nitrogen than others that have less, so it seems probable that the cellulose can be used to a certain extent as a source of energy.

The food constituents that seems to have the most noticeable effect on the occurance of azotobacter is lime. Christensen (16) says that their distribution is determined by the basicity or $CaCO_3$ content of the soil. Severin and Helene Krzemieniewski (21) found the azotobacter much more numerous in limed soils and also much

whether the soil had been fertilized with nitrogen or not. The limed soils showed fixation in ten days of 16.17---18.39 mg. while unlimed showed 6.83--7.47 mg. They found the nitrogen content in general always greater in the limed soil. Ashby (22) found the same results in the Rothamsted soils.

Bottomley (23) took limed garden soil and unlimed garden soil sterilized in an autoclave; inoculated both in the same manner with azotobacter and Pseudomonas radicicola, and in the limed soil there was an increase of 35 mg. as against 25 in the unlimed. Gerlach and Vogel (11) made up media without lime, P_2O_5 and K and got no growth. They inoculated flasks where neither K nor soda were added and here got a limited amount of growth. It may be that in the latter case traces of K and soda were taken from the glass of the container.

The beneficial action of the lime seems to be in its alkalinity and chemical action on the other constituents. Christensen (16) states that P_2O_5 and lime are essential to the decomposition of mannit. In an experiment he used soil from a plat which had been fertilized for 12 years with $NaNO_3$ without liming, and found the P_2O_5 exhausted. When this soil was used to inoculate a culture solution of mannite, $CaCO_3$ and K C L, no reduction followed. Krainskii (15) found that $NaCO_3$ was more favorable to azotobacter growth than $CaCO_3$, indicating that the benicicial action of lime was due simply to its alkalinity. Lipman (24) says that the calcium carbonate increased the amount of nitrogen fixed by azotobacter either directly by stimulating growth or indirectly by making more P S or Mg. available.

The value of inoculating fields with nitrogen fixing bacteria

for the sake of the fertilizing value is some what problematical. Experiments have given results both for and against. Stoklasa (25) inoculated pots of soil which had been adequately supplied with mannite or dextrose and lime. The number of organisms were increased and the yield and quality of the crop improved. He tried it on oats, beets and potatoes. Stranak (26) reported an increased yield of oats, potatoes etc. on inoculation. Bottomley (23) experimented with pots of sand supplied with phosphates, potash and lime and planted to oats. These gave a 76% larger yield where the plants were treated with a mixed culture of azotobacter and Pseudomonas Radicicola. In field experiments with barley the seeds were treated with this mixed culture. The plats thus treated gave a 13.6 % greater yield than the untreated. He got still higher increases in root plants. He found that $CaCO_3$ must be present in all cases to get effective work from the bacteria. Lipman (27) in field experiments could get no appreciable increase in crop yields. Heinze (18) in field experiments on fallow soil was able to get an increase in nitrogen without inoculation, by careful attention to thorough cultivation, aeration, addition of humus, lime and phosphates. This later is rather the most practical method.

The purpose of this thesis is to make a study of the occurance, fixation power and biological features of the azotobacter of Idaho Soils.

The method of determining the occurance and fixation powers of the azotobacters was by growing them in culture media. When a sample of soil was to be tested; usually four, or five-gram samples were weighed out and each put into a flask containing the

mannite media recommended by Ashby (22). It was of the following composition:--

Mannite 20 gr.

Monopotassium Phosphate 0.2 gr.

(Neutralize this first in solution and then add to other ingredients in solution).

Mg. SO_4 0.2 gms.

Na Cl 0.2 "

Ca SO_4 0.1 "

Ca CO_3 5.0 "

Dist. Water 1000 CC

To make agar 1.5 % agar was added to the above.

Of the four samples two were immediately sterilized either by steaming in the autoclave at 120°C for ten minutes or by adding ½cc of formalin. The remaining two were kept at 28°. They were allowed to grow about three weeks or until the mannit had all disappeared. Microscopic examinations were made every few days to determine the presence of mannit. This could be determined by the presence of the characteristic needle like or rhomboid crystals of mannit; Examination also was made here for presence of the azotobacter. At the end of the incubation period the cultures were Sterilized either by autoclaving at 120 ° for ten minutes or by the addition of ½cc of formalin. The contents of the flasks were then analysed for total nitrogen by the Kjeldahl method. The nitrogen analyses were made by Mr. Fishburn of the Agricultural Chemistry Department. The method used for the determination of the nitrogen was Gunnings modified method found on page 7 of Bul. 107, Bureau of Chemistry.

An experiment was made to determine whether or not the heating

Plate I Ashbys solution inoculated with five grams of soil. Incubated 11 days at 28° C. Showing growth with different soils. Flask on the right shows the characteristic blackening. The other flasks become darker with age.

(Original)

in the autoclave caused any loss of nitrogen. Soils were taken from the garden north of Morrill Hall. Eight samples of five grams each were taken and each put into a flask containing 50 cc of Ashbys solution. Four of these samples were autoclaved at 120° C for ten minutes. All eight were immediately analysed for total nitrogen.

Sterilized	Unsterile.
6.59 mg. N	6.53 mg. N
7.02 " "	6.59 " "
6.53 " "	6.59 " "
7.02 " "	7.65 " "

This showed that there was no appreciable loss in nitrogen as a result of the heating.

Four samples of soil containing a rather high percentage of nitrogen were used to duplicate the preceding experiment with the exception that ½ cc offormalin was used instead of heating. The following results were obtained:-

Sterilized with Formalin	Unsterile.
6.04	6.32
5.90	6.04

The differences are so slight that they have probably been produced by the difference in samples. The killing of the bacteria by formalin caused no appreciable loss in nitrogen.

Station Plats.

In this experiment soils came from fertilized plats on the station farms. These plats were being fertilized every three years and had just received their second application. Different applications were being made to each. of the plats as follows:-

Plat 1 had received N
" 2 " " P
" 3 " " Potash
" 4 " " Lime
" 5 was a check
" 7 had received N and K
" 8 " " P and K
" 9 " " N P K
" 11 " " L N P K

Soils from these plats h d been potted in 5 gal pots for a nitrification experiment. They had been kept in the greenhouse from Dec. 15, 1910 until the samples for nitrogen fixation were taken on Feb. 14, 1911. During this time the water content had been kept approximately at 19-21% by the addition of distilled water

Analyses for nitrates were made every two weeks. During this time the content of nitrates had increased materially:-

No. of Plat	NO_3 at Beginning of Experiment. Parts per 1,000,000 dry soil.	NO_3 at time fixation samples were taken. Parts per 1,000,000 dry soil.
1	61.5	97.4
2	34.6	86.6
3	32.2	90.4
4	51.9	129.4
5	37.0	83.9
7	30.4	114.6
8	23.2	106.8
9	45.6	133.9
11	36.4	96.1

On the Feb. 14, 1911 the samples to determine the fixation of nitrogen were taken. Two five gram samples of each soil were taken and each placed in a flask containing 50 cc of Ashbys solution. One of these flasks was sterilized in the autoclave and the other incubated at 30° C for twenty days. On March 6, 1911 analyses for total nitrogen were made.

Nitrogen fixation in Soils of Station Plats.

Plat.	How Fert.	Before	After	Increase
1	N	10.81 mg. n	12.85 mg n	2.04 mg N
2	P	10.18 " "	12.07 " "	1.89 " "
3	K	9.76 " "	11.65 " "	1.89 " "
4	Lime	10.25 " "	13.27 " "	3.02 " "
5	Check	9.69 " "	10.95 " "	1.26 " "
7	N & K	9.69 " "	11.44 " "	1.75 " "
8	P & K	10.53 " "	11.65 " "	1.12 " "
9	N & K	10.81 " "	14.74 " "	3.93 " "
11	L N P K	9.76 " "	11.58 " "	1.82 " "

The gain in nitrogen content is the heaviest in plat 4 fertilized with lime and in plat 9 fertilized with N P & K. The fixation in all these soils was very low. The cause for this may be that the soils having undergone strong nitrification in the greenhouse without loss of nitrogen through growing plants or drainage, had become so filled with nitrates that the growth of the nitrogen fixing bacteria was inhibited; and also the azotobacter may have attained their maximum growth and were now decreasing.

Creek Meadow.

The creek meadow is located north of Morrill Hall in the bottom along Paradise Creek. It is of a sandy and gravelly nature

and is subject ot overflows from the creek. A large compost heap stood within 10 rods and on higher ground than the place where samples were taken. The field is in pasture and has been for years. Samples were taken at four different times, Feb. 28. Mar. 15, April 5 and May 24th. The soil was taken from a depth of 6 inches.

At the time the first sample was taken the weather had been about 10°F above zero for several days; the top of the soil was frozen and covered with four or five inches of snow. The samples were handled in the usual method and were allowed to grow 25 days in the Ashbys solution.

Fixation of Nitrogen in Creek Meadow Soil.

Before	After
4.00 mg. N	10.18 mg. N.
4.00 " "	10.50 " "

The amount of nitrogen fixed was respectively 6.18 and 6.50 mg.

The second sample was taken Mar. 15; after the first sample had been taken the snow soon disappeared. it rained some and the creek overflowed the place of sampling. The second sample was taken about a week after the overflow and the soil was fairly well drained. The results obtained were as follows:

2nd. Determination of Nitrogen fixation of Creek Meadow.

Before	After
3.79 mg. N	10.? Lost by Chemists.
3.79 " "	9.19

The fixation amounted to 6.40; this was about the same fix-

ation as in the first sample.

The third sample was taken April 3; the weather had been cold and rainy for a week or two. The moisture content of the soil was 15% and the temperature 9° C.

3rd. Determination Nitrogen Fixation of Creek Meadow.

Before	After.
4.42 mg	9.76 mg
3.99 "	9.90 "

The fixation at this determination was the lowest recorded altho not differing much from those made before.

The fourth sample was taken on May 24th. The weather had been warm.

4th Determination of Nitrogen Fixation in Creek Meadow.

Before	After.
3.37 mg.	12.42 mg.
3.37 "	12.70 "

The fixation in this determination was 9.05 and 9.33 respectively. This was the highest fixation recorded for Creek Meadow. This high fixation resulted probably because the weather was warm and had raised the temperature of the soil, thus approaching the optimum temperature of the azotobacter. The azotobacter than probably became more active and greater numbers resulted, thus giving a greater fixation.

Boise Valley Brown Spot Soil.

Soil was received from Boise. The soil had been taken from a so called brown spot; these brown spots would not support growth of vegetation.

Determination of Nitrogen Fixation.

Before	After
1.89 mg.	10.18 mg.
1.89 "	10.95 "

The nitrogen content in the first place was very low but as this is a characteristic of the Boise Valley soils it is not unusual. The bacteria in this soil were very vigorous as in six days a strong growth and heavy scum was evident; In ten days all the mannit had been used up. The fixation was larger 8.29 and 9.06. It is evident that the cause of its unproductiveness not its lack of fixative power.

Boise Valley Island Soil.

This soil was taken from an island in the Boise river; the soil is a sandy one, containing a good deal of organic matter. The organic matter is to a great extent undecomposed.

Determination of Nitrogen Fixation in Island Soil.

Before	After.
8.28 mg.	15.44 mg.
8.21 "	14.60 "

Kuna Soil.

This soil was received from Kuna, Idaho. The soil is from sage brush land that had never been cropped. It was a fine volcanic dust soil, light in color.

Before	After
4.02 mg.	8.49 mg.
2.87 "	12.84 "

Caldwell Plats.

Soils for this experiment were taken from the plats at the substation in Caldwell Idaho. The soils were of three var-

Those labeled "Ordinary" were of the usual type of fertile south Idaho soils. Light in color and low in nitrogen. Those labeled slick were taken from light colored spots where vegetation either will not grow or do so very poorly. That labeled "Black alkali" was gathered from along a ditch. This soil does not support vegetation and after application of water a black incrustation appears on the surface.

Three samples all labeled "Ordinary 1st. ft." were composited and treated in the usual manner for nitrogen fixation. The action was rapid, the scum appearing in a few days and the mannit disappearing in 14 days. The cultures were then analysed.

Determination Nitrogen Fixation Caldwell Ordinary 1st. Ft.

Before	After.
3.23 mg.	10.25 mg.
3.61 "	12.78 "

The fixation varied from 7.64 to 7.55.

Three samples all labeled "Ordinary 2nd. Ft." were composited and nitrogen fixation test made. The action in this was present but not as strong and rapid as in the first foot, one analysed after 14 days, other after 20. days.

Determination Nitrogen Fixation Caldwell Ordinary 2nd. Ft.

Before	After
2.32 mg.	7.16 mg.
2.25 "	8.56 "

The fixation in this varies from 4.84 to 6.31. The initial nitrogen and the fixation in this soil was not as high as in the first foot. This is undoubtedly caused by there being less bacterial action in the 2nd. foot.

Soil from this same ordinary soil but on which alfalfa was growing was tested for nitrogen fixation. Two samples were composited for this. One analysed after 14 days, other after 20 days.

Determination of Nitrogen Fixation in Caldwell "Ordinary Alfalfa Soil" 1st. Ft.

Before.	After
4.70 mg.	12.21 mg.
4.49 "	10.95 "

The fixation varies from 6.25--7.92. The amount of fixation in the soil cropped with alfalfa was lower than the fixation of the uncropped. The soil from the cropped plats had a higher per cent of nitrogen.

The Pseudomonas Radicicola may have had some part in the fixation as it has been found by G. E. Gage (28) and others, that these bacteria will fix some nitrogen when grown in pure cultures.

The second foot was tested in the same manner and analysed after 20 days growth.

Determination of Nitrogen Fixation in "Caldwell Ordinary Alfalfa" 2nd ft.

Before	After
3.37 mg.	7.16 mg.
3.30 "	6.11 "

The fixation was from 2.74 to 3.86.

Slick Soils.

Two samples labeled "Slick 1st. foot", were composited and tested for fixation. The action in one flask seemed stronger than in the other. They were analysed after 20 days growth.

Determination of Nitrogen Fixation in "Caldwell Slick 1st. Foot"

Before	After.
2.88 mg.	11.51 mg.
3.09 "	8.55 "

Fixation varied from 5.46 to 8.73.

Determination of Nitrogen Fixation in Caldwell "Slick 2nd. ft."

Before	After.
2.39 mg	7.30 mg.
2.53 "	Lost in Chemists Analysis.

Fixation varied from 4.77 to 4.91.

The fixation in the slick soils does not seem to differ materially from that of the ordinary soil. Samples of slick soil from plats which were cropped with alfalfa were also tested.

Determination of Nitrogen Fixation in Caldwell "Slick Alfalfa 1st. foot."

Before	After
3.16 mg.	7.86 mg.
3.61 "	4.84 "

The fixation in this soil varied from 1.23 to 4.70.

This was lower than in the uncropped. This is the same result as was noted in the ordinary soils.

Determination of Nitrogen Fixation in Caldwell "Slick Alfalfa 2nd. foot."

Before	After
Lost in Chemists Analyses	13.62 mg.
2.18 mg	5.62 "

"Black Alkali."

The sample labeled "Black Alkali" was tested for nitrogen fixation. The liquid of the Ashbys' solutions in the flasks was

There was a strong action in these pots, the surface becoming covered with bubbles and scum within six days. These were analysed after 20 days

Determination of Nitrogen Fixation in Caldwell "Black Alkali."

Before	After
6.18 mg	10.67 mg.
6.32 "	Lost in Chemists Analyses.

These determinations showed a high initial content of nitrogen. This content is much higher than that generally found in soils from Southern Idaho. The very best garden soils of the Palouse Country have about the same amount. The fixation of 4.35 to 4.49 is not especially high. Headden (29) in his studies of the so called"Black Alkale" spots in Colorado has found them very high in nitrates, in fact so much so that it killed the vegetation. Headden(29) found as high as 6.54% of Sodium Nitrate in these spots. He has attributed this to the action of azotobacter. In this experiment we do find an especially high nitrogen content for that section of the country, but not high enough to cause the death of the plants. Also the azotobacter in these samples were not especially vigorous. More work on this is necessary to ascertain whether or not the cause in this case is the same as that in Colorado.

Gooding Soils.

Soil samples were taken from depths of 1' 2' 4' and 6' from plat 19 of the Gooding substation experiment farms. This plat had been very heavily irrigated, receiving for the growing season 2½ feet of water. The samples were handled in the usual manner. After sixteen days the flasks were analysed. At this time the flasks of the first foot showed evolution of gas and formation of scum. The second foot showed a few bubbles but no scum. No action was noticeable in the flasks inoculated with the soils of

of the other depths.

Determination of Nitrogen in the Gooding Plat 19.

	Before	After
1st. ft	3.72 mg	12.78 mg.
	3.61 "	Lost
2nd. ft.	2.81 "	3.37 "
	2.81 "	2.80 "
4th. ft.	1.40 "	1.40 "
	1.47 "	1.61 "
6th.ft	1.05 "	1.26 "
	1.33 "	1.26 "

The sterile soils show a decrease of nitrogen with increase in depth. The nitrogen fixed in the flask containing soil of the first foot, was 9.06 mg.; in the next foot, one flask showed an increase of .56 mg. the other none, the remaining ones showed no increase. This showed that the azotobacter are present practically only in the first foot. In other soils tested it was usually found that although the greatest amount of fixation was in the first foot, some was also found in the second. In this case, the plat had received excessive irrigation and it may be that the azotobacter could only get sufficient aeration for life in the first foot.

Alberta Soil.

Two Soils were received from Alberta, Canada; one was a surface soil and the other taken 24 inches below the surface. They were handled in the usual manner. They showed a slight frothy scum after growing for sometime.

Surface Soil From Alberta.

Before	After
4.42 mg	11.86 mg.

The above showed a fixation of 7.44 mg. The soil taken at a depth of twenty four inches below the surface was very dark and contained many rootlets so many in fact, that a sample could not be obtained without having some of them, this made the nitrogen analyses high.

Alberta Soil 24 inches below Surface.

Before	After.
20.14 mg	23.86 mg.

The above fixation showed an increase of 3.72 mg. of nitrogen.

The following table is a summary of all the samples tested for nitrogen fixation.

Table of Nitrogen Fixation in Soils Analysed.

Soil		Before	After	Amt. Fixed.
Station plats	Fert.			
Plat 1	N	10.81 mg.	12.85 mg	2.04 mg.
" 2	P	10.18 "	12.07 "	1.89 "
" 3	K	9.76 "	11.65 "	1.89 "
" 4	Lime	10.25 "	13.27 "	3.02 "
" 5	Check	9.69 "	10.95 "	1.26 "
" 7	N & K	9.69 "	11.44 "	1.75 "
" 8	P & K	10.53 "	11.65 "	1.12 "
" 9	N P K	10.81 "	14.74 "	3.93 "
" 11	L N P K	9.76 "	11.58 "	1.82 "
Creek Meadow	Feb. 28	4.00 "	10.18 "	6.18 "
" "	"	4.00 "	10.50 "	6.50 "
Creek "	Mar. 15	3.79"	Lost.	
" "	Mar. 15,	3.79 "	9.19 "	6.40 "
" "	Apr. 3		9.76 "	5.51 "
" "	Apr. 3	4.25 "	9.90"	5.65 "
" "	May 24,	3.37 "	12.42 "	9.05 "
" "	May 24,	3.37 "	12.70 "	9.33 "
Boise Valley	Brown Spot	1.89 "	10.18 "	8.29 "
" "	" "	1.89 "	10.95 "	9.06 "
" "	Island	8.24 "	15.44 "	7.20 "
" "	"	8.24 "	14.60 "	6.36 "
" "	Kuna	4.02 "	8.49 "	4.47--9.97 mg
" "	"	2.87 "	12.84 "	

Soil	Before	After	Amt. Fixed
Caldwell Ordinary 1st ft.	3.42 mg	12.78 mg.	9.36 mg.
" " " 2	3.42 "	10.25 "	6.83 "
" " 2nd ft	2.28 "	7.16 "	4488 "
" " " "	2.28 "	8.56 "	6.28 "
Caldwell Ordinary Alfalfa 1st"	4.59 "	12.21 "	7.62 "
" " " " "	4.59 "	10.95 "	6.36 "
" " " 2nd "	3.33 "	7.16 "	3.83 "
" " " " "	3.33 "	6.17 "	7.78 "
" Slick 1st. ft.	2.98 "	11.51 "	8.53 "
" " " "	2.98 "	8.55 "	5.57 "
" " 2nd. "	2.46 "	7.30 "	4.88 "
" " " "	2.46 "	Lost	------
" " Alfalfa 1st. ft.	3.38 "	7.86 "	4.48-"
" " " " "	3.38 "	4.84 "	1.46 "
" " " 2nd. "	Lost	13.62 "	
" " " " "	2.18 "	5.62 "	3.44 "
" Black Alkali	6.25 "	10.67 "	4.42 "
" " "	6.25 "	Lost	-----
Gooding 1st. ft.	3.66 "	12.78 "	9.06 "
" " "	3.66 "	Lost	----
" 2nd "	2.81 "	3.37 "	.56 "
" " "	2.81 "	2.80 "	.01 "
" 4th "	1.43 "	1.40 "	.03 "
" " "	1.43 "	1.61 "	.18 "
" 6th "	1.19 "	1.26 "	.07 "
" " "	1.19 "	1.26 "	.07 "
Alberta Surface	4.42 "	11.86 "	7.44 "

These analyses show that the azotobacter are well distributed in a large variety of soils. All the soils except those taken from depths of 4 and 6 feet showed some fixation. The analyses also show that the action of the azotobacter is confined to the first two feet of soil. Even in this the action is quite weak in the second foot.

STUDIES OF BIOLOGICAL FEATURES IN PURE CULTURE.

Method Of Isolation.

The method used to isolate and obtain a pure culture of azotobacter was as follows; A five gram sample of the soil from which the azotobacter was to be isolated was inoculated into a flask containing 50cc C of the Ashbys' solution. This was allowed to grow until scum appeared on the surface of the solution. A piece of this scum was then picked off with a sterile platinum needle and shaken up in a tube of melted Ashbys agar at 42° C. Transfers were then made to other tubes of Ashbys' agar and the contents plated. After growing for several days the colonies of azotobacter could be picked off onto slopes of Ashbys' Agar. This method was used and an organism closely resembling the azotobacter chroococcum (Beijerinck), was isolated from the Boise Valley Brown Spot soil. This organism was used in all the pure culture work.

Description of Colonies.

The appearance of the colonies of azotobacter on the Ashbys' agar plates is very characteristic. After growing for several

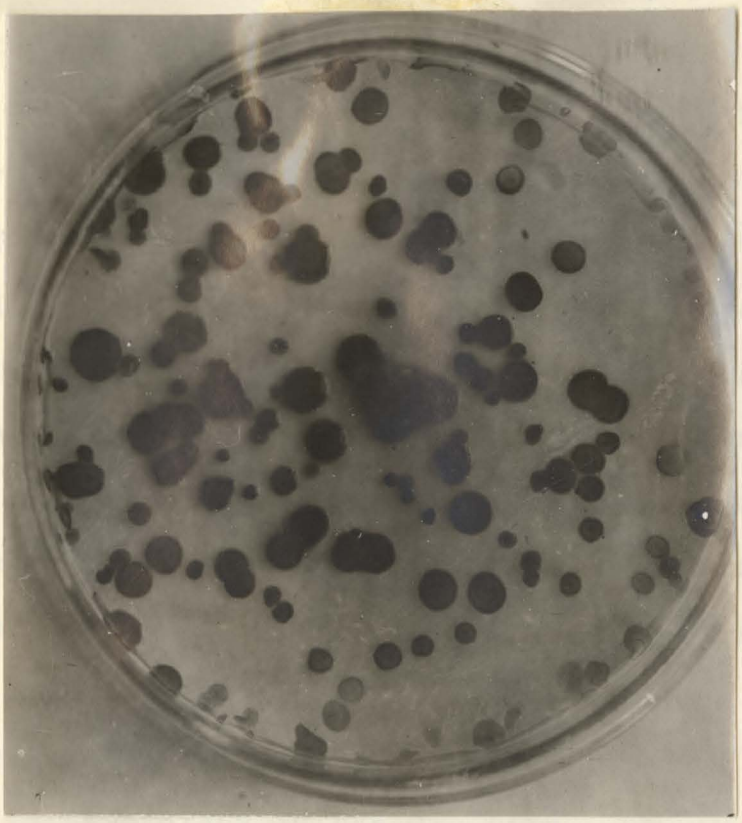

Plate II. Showing colonies of Azotobacter on Ashbys agar. Grown 15 days at room temperature.

(Original)

days in a plate containing but few colonies they attain a size of over one fourth of an inch in diameter. They seem to grow out in concentric rings. The colonies are milky white in appearance and a clear transparent space forms around them due to the dissolving of the surplus magnesium carbonate contained in the medium. After standing for some time longer and as the agar begins to dry the colonies darken and finally turn dark brown or nearly black. The illustration Plat II shows colonies which have turned dark. When these colonies are picked off onto Ashbys' agar abundant growth appears in twenty-four hours at room temperature. At first this growth is white and glistening; later on it gets older and drier, the streak darkens and finally turns black. Plate III shows three cultures of different ages on Ashbys agar slopes. A is a young culture. B is nine or ten days older, while C is about a month old.

Morphology of the Organism.

The azotobacter isolated was a large organism spherical in shape and usually between two and three microns in diameter. They, however, vary quite widely in size; one cover glass preparation showing many different sizes. The organism is found singly and in pairs. The cell appears to contain fat globules. Some trouble was experienced in making cover glass preparations of this organism as they seemed to disintegrate if strongly heated when being fixed. The best preparations were obtained when the film was fixed by holding over steam.

Physiological and Cultural Characteristics.

The organism was grown in the various media for the purpose of determining the group number according to the method recommended by the American Bacteriological Association.

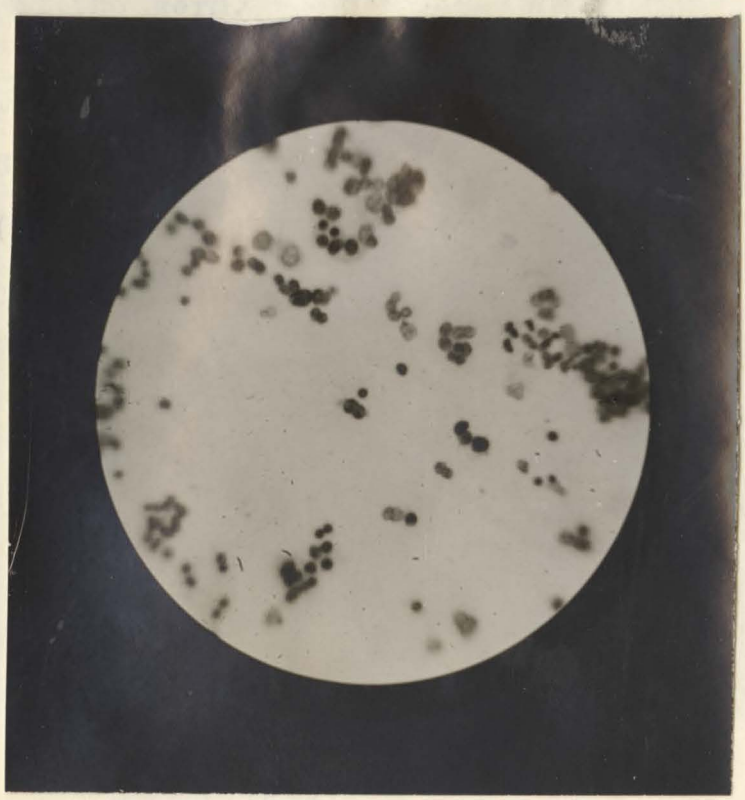

Plate IV. Photomicrograph of Azotobacter. Stained with carbol fuchsin. X 1000

(Original)

The group number of this germ is 2123333*33 on the following chart the (*) sign indicates the physiological or morphological characters of the germ.

TABLE NO. I.

A NUMERICAL SYSTEM OF RECORDING THE SALIENT CHARACTERS OF AN ORGANISM.

(Group Number)

****

1ØØ.	Endospores produced	
200.	Endospores not produced	*
10.	Aerobic (Strict)	*
20.	Facultative anaerobic	
30.	Anaerobic (Strict)	
1.	Geletin liquefied	
2.	Geletin not liquefied	*
0.1	Acid and gas from dextrose	
0.2	Acid without gas from dextrose	
0.3	No acid from dextrose	*
0.4	No growth with dextrose	
.01	Acid and gas from lactose	
.02	Acid without gas from lactose	
.03	No acid from lactose	*
.04	No growth from lactose	
.001	Acid and gas from saccharose	
.002	Acid without gas from saccharose	
.003	No acid from saccharose	*
.004	No growth with saccharose	
.0001	Nitrates reduced with evolution of gas	
.0002	Nitrates not reduced	
.0003	Nitrates reduced without gas formation	*
.00001	Fluorescent	
.00002	Violet chromogens	
.00003	Blue "	
.00004	Green "	
.00005	Yellow "	
.00006	Orange "	
.00007	Red "	
.00008	Brown "	
.00009	Pink "	
.00000	Non-chromogenic	
.000001	Diastatic action on potato starch, strong	
.000002	Diastatic action on potato starch, feeble	
.000003	Diastatic action on potato starch, absent	*
.0000001	Acid and gas from glycerine	
.0000002	Acid without gas from glycerine	
.0000003	No acid from glycerine	*
.0000004	No growth with glycerine.	

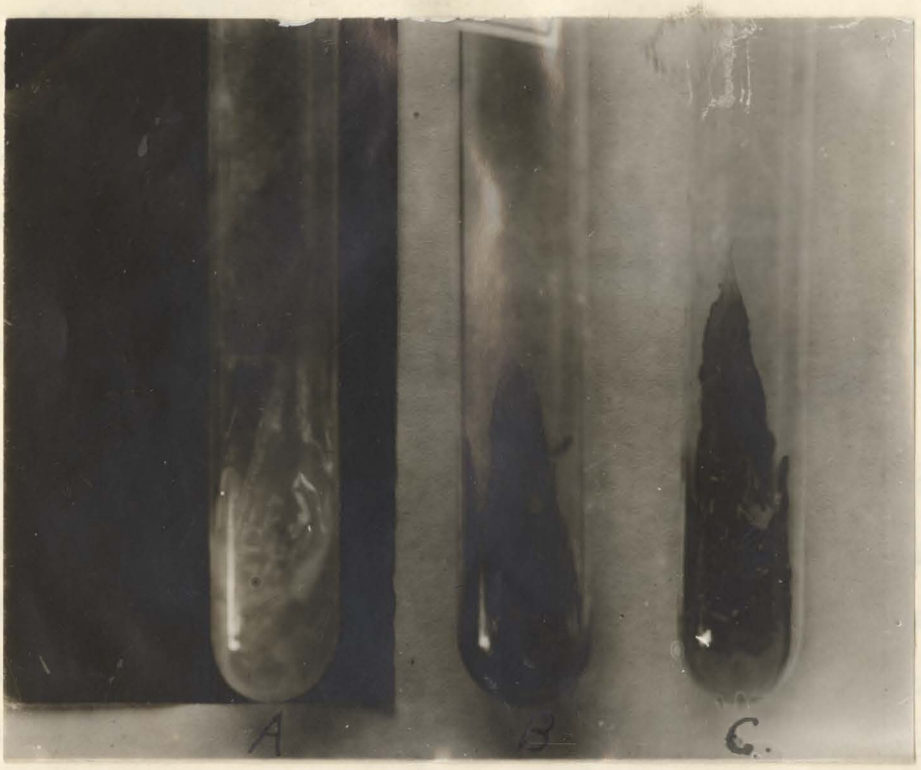

Plate III. Azotobacter growing in pure culture on Ashby agar. The growth becomes black upon age and desiccation of the medium.

(Original)

In the fermentation tubes the best growth was obtained in the dextrose bouillon and the least in the saccharose. The number for chromogenesis could not be given because the color of the streak at first is white and later becomes black. The organism is gram negative. Its growth in bouillon produces turbidity and sediment but no scum. The growth on plain naturient agar was fairly abundant but not as heavy as on Ashby's agar; it was shining and on aging the streak did not become black as on the Ashbys but became brown. The colonies on gelatin plates were irregular in shape, but not unusually large. In the gelatin stab there was surface and needle growth. On potato media the growth was entirely absent. The organism grew at 37° C. The only action it had on the litmus milk, was to turn it intensely blue. The azotobacter in nitrate solution, gave reduction, in fact it acted as a denitrifier. When nitrates are present in quantity the azotobacter do not fix nitrogen, but instead they use the nitrates in the solution for food. Stoklasa (19) and Stranak (26) found that nitrates and NH_3 were omvariably formed when $NaNO_3$ was used in culture media. No indol was formed in Dunhams solution. Fermentation tubes of Ashby' solution were prepared and inoculated; good growth resulted but no gas. Cohn s and Uschenskys solutions and agars failed to show any growth.

Effect of Drying on Azotobacter.

For this experiment samples of the Boise Valley Brown Spot soil were used. This soil had been in the laboratory at room temperature for one hundred and fourteen days. The soil it was practically desiccated. Four five-gram samples were taken; two sterilized and theother two kept at room temperature. Growth started in several days and in eight days the samples were analysed.

Before	After
-----	7.44 mg.
1.68 mg	10.53 "

The fixation in one flask was 5.76 mg in the other 8.85 mg. This same soil had been tested for nitrogen fixation when it was first received into thelaboratory. At this time a fixation of 8.29 mg. and 9.06 mg had been obtained. It is evident that this long continued drying had not affected the nitrogen fixing ability of the bacteria. Ashby (22) found that after keeping soil for a year air dried in a bottle in the laboratory he still got a good growth on inoculating the soil into the culture media.This fact aids the distribution of the azotobacter as they can be carried by winds long distances without being killed.

Growth in Sand Culture.

Forty grams of sand that had been washed and dried, and passed through a 1 m m sieve was placed into each of seven 150cc flasks, 20cc of Ashbys solution, was put into each flask and the flasks sterilized in the autoclave at 15 lbs. pressure for 10 minutes. A heavy suspension of azotobacter was made in a water blank and a loop full was inoculated into each of six flasks. The growth was very rapid in these cultures appearing in 2 or 3 days.

Two of the sand cultures were examined after six days. No

mannit was found; many bacteria were present. The surface of the slope had become covered with a glistening colorless slime. The fluid at the foot of the slope had become a gelatinous mass of azotobacter. In a couple more days the slime covering the slope turned black. After nineteen days two more were analysed; at this time the surface instead of being black and glistening had begun to dry up.

Before		After
0.00	6 days	4.14 mg. N.
0.00	"	3.93 " "
	19 days	4.49 " "
	"	Lost.

Milligram of nitrogen fixed per gram of Mannite used;

6 days	10.35 " "
"	9.82 " "
19 "	11.22 " "

Rate of Fixation in Pure Culture.

A loop of azotobacter was picked from an Ashbys agar slope and shaken up in a 9cc water blank. This was allowed to stand for fifteen minutes and then a loopful of the liquid was inoculated into each of eighteen flasks of Ashbys solution. The flasks were then incubated at 30° C. Flasks were taken out at intervals and analysed for total nitrogen.

Rate of Fixation.

Age of Culture.	Total Nitrogen content.
Check	0.00 mg.
2 days	6.52 "
2 "	6.52 "
3 "	7.02 "
3 "	6.52 "
4 "	7.02 "
4 "	8.53 "
6 "	8.53 "
6 "	8.03 "
13 "	11.04 "
13 "	11.04 "
20 "	11.93 "
20 "	15.44 "
27 "	15.44 "
27 "	14.74 "
34 "	16.88 "
34 "	20.36 "

"

In pure cultures a heavy scum does not form over the surface of the flask as it does in impure cultures.

Summary.

I. Azotobacter were well distributed in all surface soils analysed.

2. The activity of azotobacter increases as the temperature of the soil rises in the spring.

3. The action of azotobacter in soil cropped with alfalfa is less than in uncropped soil.

4. The so called "Black Alkali" soil has higher initial content of nitrogen than other soils of the same region but fixation of nitrogen in these soils in Ashbys solution was no higher than in the soils.

5. Active azotobacter, are present in greater numbers in the first foot of soil; a few are present in the second foot and none are found below this depth down to six feet.

6. Group number is 212.3333*33.

8. The production of the dark pigment is hastened by the drying of the media on which the organism is grown.

9. Drying of the soil containing azotobacter does not affect their nitrogen fixing power.

10. In sand cultures the growth is much more rapid and abundant, probably because of the better aeration.

11. Fixation of 20.36 mg. per gram of mannit was obtained in a pure culture growing in Ashbys solution.

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