Bulletin 179 June 1911

The Agricultural Experiment Station

OF THE

Colorado Agricultural College

BACTERIOLOGICAL STUDIES OF THE FIXATION OF NITROGEN IN CERTAIN COLORADO SOILS

BY

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PUBLISHED BY THE EXPERIMENT STATION
FORT COLLINS, COLORADO
1911

The Agricultural Experiment Station

FORT COLLINS, COLORADO

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BACTERIOLOGICAL STUDIES OF THE FIXATION OF NITROGEN IN CERTAIN COLORADO SOILS.

By Walter G. Sackett.

Somewhat over a year ago, Dr. Headden called my attention to the extremely large quantities of nitrates present in certain Colorado soils, stating at the time that these nitrates were frequently associated with a brown discoloration of the soil, and that this color was often confined to well defined areas varying in size from three feet in diameter to an acre or more; furthermore, that these so called "brown spots" were not fixed, inert quantities related to some recognized geological formation, but that they were alive, and in the process of making as evidenced not only by the rapid progress with which the then existing spots were spreading, but also by the almost continual appearance of new spots both in old and new localities.

Dr. Headden has been studying our alkali soils and drainage waters for the past sixteen years, and he tells me that complaints of "brown spots on which nothing will grow" have been common, but more so during the past five years; reports have been received from the cantaloupe growers that their melons are deteriorating in quality without any assignable cause; truck gardens, alfalfa, oat, barley and sugar beet fields have been developing barren patches where a uniform stand was always obtained in former years; in some parts of the state, the sugar content of the sugar beets as well as the purity and tonnage have fallen off until it is a ponderous question with the farmers and sugar factories whether the growing of sugar beets in those localities is any longer a profitable industry; but equally serious, if not even more so than any of these, is the destruction which is being wrought in some of the apple orchards of Colorado. Newly set trees, trees that have just come into bearing, and trees that are fifteen to twenty-five years old, in fact, trees of all ages, seem to suffer alike. It is not an isolated tree here and there that has died, but thousands, representing many acres of orchards in widely separated districts, have perished during the past two seasons.

When one is brought face to face with facts of such tremendous economic importance as these, he can scarcely fail to be impressed with the deplorable condition of affairs, and is forced to the position that something out of the ordinary is taking place, and that it is not without a cause.

With reference to the occurrence and distribution of the nitre areas, Dr. Headden gives the following in Bulletin 155 of this station:

"This trouble was not confined to any one section, but was common to several sections of the state. While it, in all probability, depends upon soil conditions, these conditions are met with in so many places that it is necessary to consider the condition rather than the soil

itself. It sometimes occurred in light and sandy loams, and sometimes in clayey soils. It is sometimes in comparatively low lying lands, again in the low lying portions of higher lands, and again on the hillsides. The road side, a ditch bank, and the cultivated fields represent the range of places in which this thing may reveal itself. There is one thing common in all of its occurrences, namely, a brown color in the surface soil. This color is less marked in the sandy soils than in the so-called adobe soils. Perhaps this is due to the presence of the deliquescent salts on the surface of the adobe soils, or more probably to the color of azotobacter films."

Fig. 1. Nitre area in an orchard showing the characteristic dark spots. Sample No. 30.

"We find the nitrates present in soils, where there is a great deal of moisture, but in places where there is too much water, the nitre does not appear. In little valleys and saucer shaped depressions in which the lower portions are too wet, there is no visible alkali, then follows a zone where white alkali abounds and above this the nitre is formed. I do not mean to say that there may not be nitre mixed with the white alkali, but that the nitre in such cases appears on higher ground than that on which the white alkali usually appears. Furthermore, it is not intended that anyone shall infer that it is only in valleys and depressions that the nitre occurs."

In driving through those districts which are suffering with this trouble, the most striking feature to one not conversant with the symptoms is the brownish, black and, to all appearances, wet condition of the soil. This can be seen along both sides of the travelled road, and often extends to the irrigating ditch, or fence on either side, and into the adjoining fields. I think of nothing which describes the color better than the appearance of soil where crude oil has been spilled, as is done frequently in orchards where oil pots have been used in heating, or, if you please, where the roads have been sprinkled with oil. A typical case of this sort is illustrated in Fig. 1, page 4. Considerable disappointment is experienced, however, when this blackened surface soil is examined for it is often found to be a dry crust, rather than a wet one, one-fourth to one-half inch in thickness, underlaid with one or two inches of material of a very mealy character, beneath which the soil looks like any other soil. Sometimes the surface is so moist as to be slippery, due, probably, to the presence of quantities of deliquescent salts. As one walks over a field in this condition and breaks through the hard crust, the sensation experienced has been likened to walking on corn meal or ashes.

Concerning the condition of the soil met with under the mealy layer, I can not go into details since Dr. Headden has treated this phase of the question very fully and completely in his publications, suffice it to say that free water is seldom found nearer to the surface than five feet, and in most cases the soil is in what would be considered a nice moist condition; again in the heavier lands, we may expect and do find them rather sticky near the surface and of a gumbo character as the water plane is approached.

The brown color often appears on the banks of the irrigating ditches, eight to ten inches above the level of the water, and along the upper edge of the irrigation furrows. Extending lengthwise of these, it manifests itself a few days after irrigation as broad bands of pigment which might easily be mistaken for manure stains, so far as color is concerned, especially if the field or orchard had been fertilized recently. It is not uncommon to find large tracts of land where the nitrates have become so abundant as to be deleterious to the crops, yet no discoloration is apparent on the surface. It is difficult to say in such instances whether no color is being produced or whether it is developing so gradually and uniformly that it can not be detected readily.

The economic outlook of this problem is, indeed, a serious one. Bushels of wheat have been planted on heavy nitre soils, and if it germinated at all, only a very small percentage ever came through the ground. Oats and barley have suffered the same fate. Corn has germinated in some fields, and made a sickly, yellow growth of six inches to a foot and then died. Sugar beets, if they grew at all, have gone to tops, while the roots have taken on all sorts of abnormal, irregular shapes, typical "tub-beets," to say nothing of the inferior quality of the beet from the sugar standpoint. Dr. Headden has collected

a great deal of data on this point which will be presented by him in due time. The money loss to the farmers in seed alone has amounted to thousands of dollars. But the orchardist has been unquestionably the heaviest loser, for not only has he been deprived of the crop for the current season, but he has also lost the trees upon which he is dependent for future crops, at least we have yet to see a single tree which has shown any indication of recovery. Added to this and worse than all, perhaps, is the utter worthless and hopeless condition of his soil for agricultural purposes. The apple, cherry, apricot and plum, all appear to suffer about equally, while the pear and peach, thus far, have exhibited marked resistance, the peach having been observed to suffer least of all.

The symptoms of excessive nitre in the soil as manifested in apple trees are so characteristic that it may be well to describe them briefly in passing. The first indication is the firing or burning of the leaves along the margins, beginning at the apex, extending rapidly along the edge, inward toward the midrib and downward toward the base until the entire leaf has turned brown. There is no occasion for anyone who is familiar with the yellowing of foliage due to lack of proper drainage to confuse this with the nitre burning, for the appearance of the leaves in the two cases is entirely distinct. Whole trees have been known to undergo this transformation in less than three weeks time. In fact, Dr. Headden reports having killed a four-year-old tree in an experimental orchard in four days by applying twenty pounds of nitrate of soda around the roots and then irrigating at once to bring the nitre into solution. In reference to the behavior of this tree, he says, "The effects were in all respects similar to those produced in other orchards" under natural conditions. If the burning of the leaves occurs early in the season, the trees will often exert a feeble effort to put forth a second crop of leaves. These are usually small, whitish leaves and inclined to be rather pubescent. Such trees, laden with one-third to one-half grown apples, seldom mature any fruit, and in all probability will be dead by spring. If the attack comes late in August or September, the chances are that the fruit will mature, but it will be undersized and of poor quality; no new leaves will be expected to appear and the old ones will cling to the twigs late into the fall. The following spring, it is very likely that an attempt will be made at leafing out, but as stated above the leaves will be small, yellowish-white and few in number, and by the middle of the season the tree will be dead.

Before proceeding further, I wish to make it perfectly clear that what I have said is not to be interpreted as applying to all of our arable land or to more than a very small percentage. While the matter is eminently serious, it by no means justifies the position that our agricultural interests as a whole are in jeopardy. Just how we shall meet these difficulties, and correct the trouble, we are not prepared to say at present, but we are hopeful. As our knowledge of the subject grows, we feel confident that remedial measures will be forthcoming

in the near future. In this connection, I may say that it is my purpose this coming season to plant several foreign grasses, reputed to be heavy nitrogen feeders, on high nitre soils with the expectation of securing some crop which can utilize the nitrogen.

In order that the reader can have a more definite idea of the amount of nitrates which have been found in some of these once arable soils, I am giving below a few figures on this point which have been furnished to me by Dr. Headden, to whom I am indebted for the soil analyses and many of the soil histories contained in this bulletin.

By way of comparison, I may say that an average amount of nitrate for our cultivated fields is from .000626 to .002005 per cent.

TABLE No. 1. Nitrates in certain Nitre Soils.

Source	Material examined	Percent water soluble	Percent nitrates in water soluble	Percent nitrates in air dried soil
Black spot in barley field.	Surface soil two inches	13.4	41.859	5.628
Young orchard	Surface soil	22.466	29.114	6.54
Young orchard	Surface soil two inches	8.23	8.173	.673
Alfalfa field	Top soil five inches	7.78	33.06	2.571
Oat field	Surface soil two inches	5.42	50.221	2.722
Orchard	Top soil 12 inches	6.51	43.57	2.837
Corn and rye.	Surface soil	4.67	7.352	.342
Old orchard	Surface soil	6.65	5.746	.382

These figures may mean more when I say that one of the above samples, which carried 2.873 per cent of nitrates in the surface foot, contained nitrates corresponding to 113,480 pounds, or 56.74 tons per acre foot; in another sample, taken to a depth of five inches, the area involved being about eight acres, sodic nitrate corresponding to 344,000 pounds or 172 tons was found in the surface five inches; in the top four inches of another eight acre tract, the equiva ent of 189,971 pounds or 95 tons was found.

With such quantities of nitre in the soil as these figures indicate, it seems hardly necessary to look elsewhere for an explanation of the death of our trees and crops.

Because of other investigations which were already under way, I was not able to take up this very interesting question until recently, except to go on occasional, hurried field trips into the districts involved. Here, I saw all that had been described to me, and, I must confess, intensified and more serious than I had imagined.

A very natural explanation for the accumulation of these nitrates, and one which may have suggested itself to the reader, would be the

concentration of the salts from the irrigation and ground waters in the surface layers of the soil. This, of course, presupposes the existence of a nitrate bearing stratum from which to derive this salt. In the first place, no such stratum or bed is known to exist within the state or neighboring states, and, in the second place, our deep well waters, ground waters and surface waters contain an insignificant and negligible quantity of nitrate.

That these spots are the remains of great herds of extinct animals which perished from some unknown cause is highly improbable, first, because the areas involved are too great; second, as mentioned before, the present spots are increasing in size, and, third, spots are appearing today in localities where the trouble has never been reported before.

For the same reasons, there is no ground for believing that these areas are nitre beds related to some established geological horizon.

Unable to account for this phenomenon satisfactorily in any of the foregoing ways, we have been forced to the one remaining possibility, namely, the formation of the nitrates *in situ*.

Having reached this conclusion only after a thorough study of all other possible causes, Dr. Headden presented the question to me as a purely bacteriological problem, amenable to bacteriological methods.

Under ordinary circumstances, I should have looked to the ammonifying and nitrifying flora of our soils as the responsible agents, but the amount of organic matter in our soils, both cultivated and virgin, is far too small to supply the organic nitrogen required for the manufacture of such quantities of nitrate. Confronted by this nitrate monstrosity on the one hand, and by the dearth of nitrogen on the other, I must confess that the question was somewhat perplexing. However, it seemed to me that the logical method of procedure was to look elsewhere than to the soil for a source of nitrogen. Quite naturally, I turned my attention to the atmosphere. If it could be demonstrated that our soils had the power of fixing atmospheric nitrogen through the agency of Azotobacter and did fix it, I felt reasonably certain that it was only a question of time until we could show that the ammonifying and nitrifying organisms were utilizing this new supply of nitrogen to build up nitrates.

With this as a starting point, I have begun my studies on the fixation of nitrogen by Azotobacter in certain Colorado soils.

SCOPE OF PRESENT WORK.

Our studies on the fixation of atmospheric nitrogen, presented herein, have not been confined to fixation in solutions alone, but have been extended to include fixation in the soil itself. Two such soil experiments are reported here, but the greater part of this data has been reserved for another bulletin.

Extended studies on the ammonifying and nitrifying efficiency of these same soils are in progress at the present time, the results of which will constitute the text for a future publication.

GENERAL METHODS.

For determining the nitrogen fixing power of the different soils in solutions, we have used the mannite solution recommended by Lipman[1] except that the tri-basic potassium phosphate, (K_3PO_4), has been substituted for the di-basic, (K_2HPO_4).

Mannite Solution for Nitrogen Fixation.

Tap water	1000.00 c. c.
Mannite	15.00 grams
K_3PO_4	.5 grams
$Mg\ SO_4$	.2 grams
$Ca\ Cl_2$	.02 grams
10 per cent solution $Fe\ Cl_3$	1 drop

This was made neutral to phenolphthalein with normal NaOH. 100 c.c. of this solution containing 1.5 grams of mannite were employed for each soil examined. It was placed in 500 c. c. Erlenmeyer flasks and sterilized in the autoclave for five minutes at 120 degrees C. These solutions were inoculated with 20 c.c. of the given soil infusion corresponding to 10 grams of the soil. The infusion was prepared by mixing 150 grams of soil with 300 c.c. of sterile physiological salt solution (.75% Na Cl), shaking the mixture for five minutes and then allowing it to stand for thirty minutes for the coarser particles to subside, after which the inoculating suspension was withdrawn with a sterile pipette. Four flask cultures were prepared from each soil, two of which were analyzed for total nitrogen immediately, and the remaining two after thirty days' incubation at 28 degrees C.

For isolating and growing our stock cultures we have employed a mannite agar of the same composition as the mannite solution with the addition of 15 grams of agar per 1000 c.c. of solution.

In isolating Azotobacter from the crude soil cultures we have depended solely upon repeated plating for obtaining cultures in a pure condition. We were troubled a great deal with a small bacillus which was almost invariably associated with the Azotobacter colonies in the original plates but by replating through three dilutions, sometimes three different times, we were able to obtain pure cultures. The intermediate glycerine solution recommended by Lipman did not prove satisfactory in our hands.

Our stock cultures of Azotobacter were isolated in March, 1910, and have been transferred every fourteen days since that time on mannite agar.

For determining the total nitrogen in the cultures and in the soils, we have used the Gunning Method modified to include nitrates as described in the Official Methods of Analysis,[2] page 8.

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1. Rept. of Soil Chemist and Bacteriologist, New Jersey Exp. Sta. 1908, p. 137
2. Bulletin No. 107 (Revised) Bureau of Chemistry, U. S. Dept. Agr. 1908.

DISCUSSION OF THE SOILS UNDER STUDY.

NITROGEN FIXING POWER IN SOLUTION.

In order that the reader may have a truer appreciation and a clearer conception of the actual conditions as they exist in the soils about to be described, it has seemed desirable to the writer to accompany each with a brief description of the field or orchard from which the sample was obtained. In several instances, the quantity of nitrate found appears to be in excess of all reason, but when measured by the damage done to vegetation, it falls easily within the limits of possibility. The converse of this proposition is equally true. If we are to witness the destruction of a forty-acre orchard in one season, we shall need to look for some such powerful agent as nitre occurring by the tens of tons to the acre foot.

SAMPLES NOS. 1, 2, 3, 4.

It is needless to say that at the beginnig of our work we encountered unforeseen difficulties. We were dealing with soils which were decidedly unlike other soils, and in which previously unheard of conditions maintained. Naturally, our first samples were taken from those spots where the trouble was unmistakably present, and there was nothing to tell us how concentrated the nitrates might become and still permit the growth of the soil flora. The results obtained with these early samples were rather disappointing inasmuch as they seemed to possess practically no nitrogen fixing power when tested out in mannite solutions. Subsequent work, however, showed that the nitrogen fixing bacteria, as well as the higher forms of plant life, had been destroyed by the nitrates.

After a little experience we were able to establish, in a general way, a relation between the appearance and the nitrate content of a soil, on the one hand, and the probable occurrence of Azotobacter on the other. This made it possible to take samples more intelligently and to avoid the extremely high nitre areas. Samples 1, 2, 3 and 4 will serve very nicely to illustrate the relation of nitrate accumulation to the presence of the nitrogen fixing bacteria. They were collected September 21, 1909.

All four of these were taken from a forty-acre tract, twenty acres of which had been in bearing orchard and the remainder in alfalfa. In 1907 barren spots began to appear in the alfalfa; brown patches, here and there, in the orchard soon became conspicuous, and the trees commenced to die. By 1909, fifty per cent of the orchard had perished along with the entire twenty acres of alfalfa, and in 1910, portions of possibly six rows along one edge and a few trees in a far corner were making their last struggle. The whole center was a barren waste with not a weed to be seen. The greater part was brownish black on the surface, glistening with crystals and to all appearances wet, but, in fact, covered with a hard, dry crust about 3-16 inches thick. Beneath this, the next 1½ to 3 inches were mealy in

character, a mixture of soil and crystals. Below this, the soil grew wet very rapidly and at sixteen inches was practically mud. There was no free water at three feet and in a nearby excavation which had been made for a cellar, over three feet deep, there was no water, yet the soil which varied from a sandy loam to a calcarious clay was decidedly wet and sticky sixteen inches below the surface. It was necessary to dig to a depth of six feet to reach the flow of ground water.

Sample No. 1 was composed of the surface crust of which 12.523 per cent was soluble in water. 19.822 per cent of this, or 1.482 per cent of the air dried soil consisted of nitrates. The flask culture did not develop a typical brown Azotobacter membrane, but rather a whitish yellow scum. This was composed mostly of rod shaped organisms with scattering Azotobacter like forms which disappeared with age. A marked gaseous fermentation of the culture solution took place, accompanied by formation of acetic acid. In thirty days, this soil fixed 1.05075 m. g. of nitrogen.

Sample No. 2 came from the mealy layer beneath the crust; 8.44 per cent of this was water soluble, 15.421 per cent of which or 1.301 per cent of the air dried soil was nitrates. A very delicate, white scum with almost no growth in the body of the liquid was all that was obtained in the culture. There was some fermentation and slight acid production with the odor of butyric ether. In thirty days, the increase in nitrogen was so small as to be practically negligible, being only .5604 m.g.

Sample No. 3 consisted of the twelfth to fourteenth inch inclusive where the ground was wet. Unfortunately I do not have the analysis of this portion of the soil, but in a nearby orchard the fourth to fifteenth inch inclusive contained .676 per cent nitrates. In all probability, my sample carried less than this since it contained a smaller amount of the rich surface material. With this, I secured the Azotobacter forms in a limited number, comparatively speaking, along with the ordinary rods which made up the patchy film and flocculent growth of the culture. Some fermentation and slight acid production with a cheesy odor were observed. An increase of 3.43245 m. g. of nitrogen was obtained after thirty days.

Sample No. 4 came from near a tree along the edge of the affected area. The soil appeared normal and the few trees close by seemed in a healthy condition. The surface two inches were removed and the second to sixth inch inclusive were collected. A typical chocolate brown, wrinkled Azotobacter membrane was obtained with this material in five days. This form was very abundant and dominated in the culture which was slightly acid and possessed an earthy odor. After thirty days, the determination of nitrogen showed an increase of 12.4689 m. g.

A comparison of the results obtained with these four samples suggests that the nitrates were so abundant in the first two that the Azotobacter had either been destroyed or so weakened in virulence that

little or no fixation could be accomplished; that the fourteenth inch sample, while rich in nitrates, did not carry enough to inhibit entirely the growth of the nitrogen fixing bacteria, and that in No. 4, the conditions were very favorable for these organisms.

SAMPLE NO. 5.

This sample was procured September 21, 1909, from an orchard between the irrigating furrows. The brown surface soil, a light clay loam, was removed and a section, including the second to sixth inch, was taken. In 1908 a few of the trees had died and the owner, believing that possibly this had been caused by lack of fertility, had given the orchard a liberal dressing of stable manure. The following spring the ground from which the dead trees had been removed, one to two acres perhaps, along with seven or eight acres of the orchard, was sown to wheat, but to the dismay of all concerned this, too, failed to grow, only a very small per cent ever coming up. During the summer, 1909, fifteen to twenty-year-old trees died by the score, beginning early in the season and continuing late into the fall. A conservative figure for the damage done this year would be the loss of 300 bearing apple trees.

1910 saw a continued spread of the burning, and already in 1911 the attack is being renewed with increased vigor.

In the culture solution, the growth took the form of a dull, almost continuous scum, with patches of white, gelatinous material here and there. There was a slight acid production with the odor of butyric ether at times. The microbic flora consisted principally of large rods, mycelial threads and many clostridium forms resembling closely, if not identical with, Clostridium pastorianum. An increase of 3.0822 m. g. of nitrogen was obtained in thirty days.

SAMPLE NO. 6.

The material for this test was taken from the top of a brown irrigating furrow in a beet field. The surface crust was removed and the next four inches used. The soil was a sandy clay and the field was in alfalfa in 1906. At this time complaints were received of the appearance of bare spots on which the alfalfa was dying out. The largest of these was horse-shoe shaped and about one-half acre in extent. The chief trouble in this instance was seepage but in 1908 the field was sown to oats, and it was not long before a number of brown patches, mealy in character, developed on the higher places. When the land was being prepared for beets in 1909, there was nothing unusual to create one's suspicion except the seepage. I visited the field in September and there were great bare spots surrounded by beets with immense tops. This is shown in Fig. 2, page 13. The stand had evidently been very poor since some of the barren places would average a half acre in area. The soil was mealy and high in nitric acid. That fall the land was sown to winter wheat and when I saw it the next summer, the whole twenty-five acres was a total failure.

My sample was secured in September, 1909, and in thirty days gave a fixation of 3.57265 m. g. of nitrogen. In culture it produced a lemon yellow membrane on the surface with a similar growth in the bottom of the flask. There was a slight acid production accompanied by a butyric, cheesy odor.

Fig. 2. Nitre spot in a sugar beet field. Sample No. 6.

SAMPLES NOS. 7 AND 8.

These samples were taken from a deserted field where the brown areas had become very numerous and extensive. The Russian thistles which had been the last inhabitants of these spots had died and left them bare. Adjoining orchards were suffering from the nitrate burning. The trouble was first noticed here in 1908 and in September of 1909 I took my two samples. No. 6 came from a brown spot, five feet in diameter and No. 7 was taken three feet outside this affected area. In both cases the surface two inches were discarded and the next four inches collected. The soil was a red, gypsiferous clay and no water occurred anywhere near the surface. In culture, these two resembled each other very closely. They produced a yellow membranous growth, with some gas, little acid, and a cheesy odor. No. 7 developed a more typical Azotobacter membrane than No. 8. This was composed largely of the characteristic Azotobacter forms and numerous other small rods and clostridia, but no brown color developed. The membrane produced by No. 8 was not as heavy as No. 7, but was made up of practically the same forms. In thirty days soil No. 7 fixed 3.01215 m. g. and No. 8, 2.87205 m. g. of nitrogen.

SAMPLE NO. 9.

All of the samples which had been collected thus far had been from either high nitre spots or immediately adjoining areas, and it seemed

to me quite desirable that we should have a specimen of soil from some locality where this nitrate trouble had not been heard of. It looked only reasonable that if the nitrates were killing apple trees, they were probably also destroying the microbic soil flora, and I wished to have a sample of what might be considered a normal soil as a check. For this purpose we obtained material from an alfalfa field where there was no history of any trouble. The ground was light, well drained, and judging from the size of the plants, it had been in alfalfa for a number of years. The surface two inches were discarded and the next four inches were taken for the sample.

In ten days a heavy, gelatinous, white Azotobacter membrane formed in the culture flask. Azotobacter was the dominant form, but other small rods were also present. There was some fermentation and an odor of rotten cabbage was developed. After thirty days the membrane was brown in color and had the physical appearance of cold grease which has hardened on top of a beef infusion and later has been disturbed and broken. This soil gave an increase in nitrogen in thirty days amounting to 10.15925 m. g. After obtaining this result and a similar one with No. 4, I felt pretty well satisfied that the true nitrogen fixing power of the soil could not be judged from a sample which was taken from either the dark crust of a nitre spot or an area where all vegetation had been dead for some time and where a chemical analysis showed the nitrates to be extremely high. I felt very confident from our work thus far that some of our soils, at least, were stocked abundantly with Azotobacter chroococcum, but it was very evident that this genus had either been destroyed or become greatly attenuated where the nitrates were excessive. I am inclined to accept the former view by way of an explanation since I have plated out crude cultures repeatedly which were made from very bad soils and have failed to obtain anything which resembled Azotobacter. Again I have had no difficulty at all in isolating pure cultures of Azotobacter from crude cultures prepared from soils which were high in nitrates, but not excessively so, and which a month later developed fatal quantities. After my experience with these two samples, I decided to take the soil for future work from areas where the nitre was just beginning to manifest itself on the vegetation and toward which the wave of nitrate destruction was advancing. I am glad to say that I was not disappointed in adopting this new way of sampling as the following experiments will testify.

SAMPLE NO. 10.

In April, 1910, Dr. Headden called my attention to the unmistakable brown stain on the irrigating furrows of a young orchard belonging to the Experiment Station. This was the first indication of the trouble that had been observed in this immediate vicinity. The color was confined to the furrows, there was no mealy condition of the soil and the trees were in perfect health. The soil was a clay loam, well drained, with gravel and ground water at 18 to 20 feet. Up to the pres-

ent time no injury has been observed in the orchard. The same brown color was very marked along the roadside where the irrigating water had been running three days previously. With this soil taken from the furrow, a yellowish brown membrane, consisting largely of Azotobacter, was obtained, and upon analysis the culture showed an increase of 7.7055 m. g. of nitrogen in thirty days.

SAMPLE NO. 11.

Sample No. 11 was obtained from an alfalfa field located on river bottom land where the water was quite near the surface. Five to ten acres of alfalfa had died and the barren spots were brown to black on the surface. The soil was a light alluvial formation and admirably suited to agriculture. The top four inches were taken for examination June 1910. In culture it developed a heavy straw colored, leathery membrane, after 48 hours, which was composed of Azotobacter cells with many large and small rods. After thirty days, an increase of 5.11365 m. g. of nitrogen was obtained.

SAMPLE NO. 12.

A complaint was received from a certain truck gardener in July 1910, stating that there were places in his garden where, for several years, he had been unable to secure a satisfactory stand. At that time, his chief trouble was with carrots and parsnips. Three to five acres were involved this season, and from the brown appearance of the surface and mealy character to the tread, the soil looked very suspicious. It was a nice sandy loam and no trouble was ever experienced in raising crops except on the barren spots. Even on these, when the plants became once established, they grew very luxuriantly. A sample consisting of the three inches of top soil, taken about ten feet from a barren place, was procured. This yielded a heavy, wrinkled, pale yellow membrane, which browned with age. Azotobacter was abundant and after thirty days our analysis showed an increase of 8.61615 m. g. of nitrogen in the culture.

SAMPLE NO. 13.

We come next to an orchard where the burning first appeared in 1909 on the apple trees. The point of particular interest in this case is the marked resistance which a block of pear trees has shown to the nitre. There are about three acres of these trees in full bearing and, although lying adjacent to a five acre apple orchard which is badly affected, and in the direct path of the nitre streak, the first injured pear tree is yet to be seen. As a matter of fact, this immunity of the pear is an occurrence of rather frequent observation. The apple orchard embraced about seven acres and the trees were all from twenty to twenty-five years old. They had been in perfect condition and yielding abundantly until the summer of 1909 when the burned leaves began to make their appearance. A section in the center of the orchard of about one half acre, succumbed that season, but before July, 1910, when I took my sample, three more acres had died and had been pulled up and the

ground planted to corn. This stand was very poor. Much of it died in the ground shortly after germination, while some that did grow, attained a height of 8 to 10 inches, with sickly yellow leaves and finally died. By the end of fall, 1910, approximately 300 trees had been taken out and consigned to the brush heap and wood pile. I have learned recently that the remaining acre and a half began dying so rapidly this spring (1911) that it, too, was grubbed out. Thus, the complete ruin of the seven acres was brought about in less than two years. The soil is a sandy loam, underlaid with gravel at 5 to 8 feet. There is no water near the surface. The characteristic brown color was plainly visible on the crests and sides of the irrigation furrows and it was from one of these that I took the top three inches of soil for examination. Azotobacter developed readily in culture with the characteristic membrane and after thirty days an increase of 4.13295 m. g. of nitrogen was secured.

SAMPLE NO. 14.

The next sample was procured from an orchard where only a few trees showed signs of firing in the early summer of 1910. I visited this place in July, 1910, and it took considerable diligent hunting to find the few scattering trees which were suffering. There were perhaps twenty in all. Today six acres of this orchard are dead from no other cause than nitre. There was no water at five and a half feet and the soil was a nice clay loam. The sample for the fixation test was taken from between two rows of trees which seemed to be as badly affected as any and included the surface three inches. The ground between the trees had been recently cultivated so that any brown stain which might have been visible on the irrigating furrows had been obliterated. There was no indication of excessive nitrates in the soil other than the burned edges of the apple leaves. Pure cultures of Azotobacter were isolated readily from this soil which gave an increase of 6.65475 m. g. of nitrogen in thirty days.

SAMPLE NO. 15.

While looking over the orchard described above I was asked to pass an opinion on some apricot trees in a neighboring orchard. They were large trees, seven in number, and affected in a most peculiar way. The foliage of the entire tree was wilted as if the water supply was cut off; the leaves had a good green color and there was a heavy set of fruit which was just beginning to ripen. A short distance from the trees, I discovered the brown color on the soil which we have come to regard as an important symptom of the nitre trouble. I set about at once to look for signs of this on the apple foliage nearby, and before I had gone far, my search was rewarded. The number of trees involved was limited to possibly a dozen and these were not burned severely. However, by the end of the season, all of these had died and were taken up, leaving about a quarter of an acre barren. There was some indication of too much water in this orchard and it is very possible that the

question of seepage should be taken into consideration here as well as the high nitrates.

A sample, taken near one of the apple trees, showed the soil to be a rather heavy clay loam. In culture it developed a characteristic white, gelatinous Azotobacter membrane and in thirty days gave a nitrogen increase of 10.15725 m. g.

SAMPLE NO. 16.

With Azotobacter as widely distributed as the foregoing sample indicated, there came the natural query, whether this genus was not indigenous to all of our soils in a raw state as well as cultivated. To determine this point I procured my next sample July 13, 1910, from the top of an adobe hill which was above all ditches and consequently was watered only by the scant rainfall. No vegetation was growing here and because of its location and inaccessibility, I have my doubts whether man had ever trod that particular soil before. It was literally raw land in the process of formation. The underlying decomposing shale from which the shallow soil was being made came to the surface in a number of places. The culture solution which was inoculated with an infusion of this material showed only a very slight growth, visible as a slight turbidity and delicate scum. In fact, this may have been due to the infecting substance itself. Microscopic examinations of the solution were made at frequent intervals but nothing which resembled Azotobacter could be detected at any time. After thirty days, there was a slight apparent increase in the nitrogen content of the culture but this was so very small, amounting to only .2802 m. g. that it could be easily accounted for by the personal equation. It was clear from the result obtained here that this virgin soil, at least, possessed neither nitrogen fixing power nor nitrogen fixing flora.

SAMPLES NOS. 17 AND 18.

After testing sample No. 16 and finding that it was practically inert so far as nitrogen fixing power was concerned, we were interested in learning what effect cultivation might have upon such a soil since many of the orchards which have been in cultivation from 15 to 20 years were set out in soil similar to this adobe shale. What is more, it is in those older orchards which have been irrigated longer and cultivated more vigorously that we find the nitre trouble making the most rapid progress. If possible, we wanted to get a soil sample from a young orchard recently set in raw adobe shale, where there had been but a limited amount of cultivation. We were fortunate in securing just such a case. At a distance of perhaps a mile from the adobe hill from which sample No. 16 was taken we found a piece of raw land which had been broken for the first time in the fall of 1909 and set to young apple trees in the spring of 1910. This was watered by a high line ditch in which the water had not been superabundant and consequently it had received but little irrigation, and that during only one season. About the only difference between this land and the adobe

hill was the difference in the physical condition brought about by cultivation to conserve the moisture. Two samples were taken, Oct. 26, 1910, from this orchard, one (No. 17) from between the rows of trees well out in the tract, and the other (No. 18) from a ditch bank where the moisture conditions should have been more favorable for the growth of the soil bacteria. In culture, neither of these soils produced any surface film and gave only a thin, white membrane in the bottom of the flask. The nitrogen determinations after thirty days indicated that the soil from the ditch bank had actually lost nitrogen while the increase with the other was so slight as to be negligible, (.35025 m. g.) If any conclusion can be drawn from the examination of samples Nos. 16, 17, 18 it would seem to indicate that our adobe shale soils both in the raw state and during early cultivation lack nitrogen fixing powers.

SAMPLE NO. 19.

The next sample was taken July, 1910, in a bearing orchard where the burning had appeared for the first time the previous year. There was no well defined area in which one could say that the trouble was worst but it was scattered throughout. In all, about two and one-half acres had been killed when I visited the ranch. The soil is a sandy loam which cultivates beautifully. It is underlaid with gravel at five and a quarter feet in whch there is a little water at times. A hole six feet deep was dug in this orchard and allowed to remain open for one year for the purpose of seeing if there was an excess of water in this soil which could be removed by proper drainage. At the end of the time stated, the hole was as dry as the day it was put down. The brown color was plainly visible on the sides and crests of the irrigation furrows and the trees were dying in a manner that we have come to recognize as specific for nitre burning. The sample from this orchard was taken from between the irrigating furrows near a tree that was just beginning to burn. In culture, there developed a heavy gelatinous, white, wrinkled membrane with scattered brown patches. Abundant Azotobacter cells were present. After thirty days, it showed an increase of 9.807 m. g. of nitrogen.

SAMPLE NO. 20.

Although I felt reasonably certain at the outset that we would secure little, if any, fixation with this soil, I was interested in learning whether, when an orchard died in a phenonemally short time, as was true here, the Azotobacter were likewise killed. There were about thirty acres altogether in the orchard, fifteen of which died between June, 1909, and July, 1910. Sample No. 20 was taken from that section which had been destroyed in 1909 and which had received no water during 1910 so that by July the surface was very hard and dry. The soil varied from a sandy loam to a clay loam with no water at six feet. In culture, a delicate, white, membranous film was formed on the surface with some flocculent growth in the liquid. A sour, earthy odor was developed. The increase in nitrogen amounted to only 2.5218 m. g. in thirty days, demonstrating again that the concentration of the

nitrates, which had proven disastrous to the trees, had also had its detrimental effect upon the nitrogen fixing flora.

SAMPLE NO. 21.

This sample, a red sandy loam, was taken in an orchard, July 1910, where the conditions were much the same as those described for No. 20. The orchard included about two acres along one side of which ran a twenty foot wash so that every chance for good drainage was afforded. The trees had begun dying here in 1910 and were about all gone by late summer. The soil was brown along the ditch banks and irrigating furrows. In culture a moderately heavy, white film, containing Azotobacter, formed on the surface of the medium and there was some butyric fermentation. The increase in nitrogen after thirty days amounted to 2.8014 m. g.

SAMPLE NO. 22.

In this sample we have one of the most severe cases of nitrate destruction which we have ever recorded. Here is a 90 acre orchard which showed the first symptoms in 1908, and today at least forty-five acres are entirely dead or will be by fall. The soil varies from a red clay to a sandy loam and there is no water at five feet. When sampled late in July, 1910, scattering trees were badly affected, but many were showing only a few burned leaves on the water sprouts. The irrigating furrows showed a light brown stain, more especially on the crests than along the sides, since the orchard had been irrigated recently and it was rather difficult to distinguish the brown color from the moist conditions of the furrow. There was little doubt at this time that the trees were in a very dangerous condition but it was hardly expected that in less than a year half of the tract with its fifteen year old apple trees would be waste land. This soil gave a heavy white gelatinous membrane composed mostly of Azotobacter cells and after thirty days the nitrogen of the culture had increased 8.89635 m. g.

SAMPLE NO. 23.

We come next to an orchard where the soil is a red clay loam. This is one of the more recent orchards to show the burning and nothing unusual was observed here until July, 1910. At this time very few of the trees were killed outright but many were in the first stages and some were in a very critical condition The area of the orchard was about forty acres and over one half of the trees are dead today. The soil showed almost no brown color when I took my sample, due possibly to the peculiar red color naturally present. I am inclined to believe that at this time the nitrates had not become extremely high or more of the trees would have been killed and we should undoubtedly have seen more of the brown stain. The culture from this sample showed almost no surface growth but a white membrane on the bottom and sides of the flask. Along with this there developed a marked foecal odor. Azotobacter was present in quantity. The increase in nitrogen in thirty days due to fixation amounted to 7.1451 m. g.

SAMPLE NO. 24.

A barley field on the top of a mesa was the next location chosen for our work. This particular tract was selected for several reasons. In the first place, the nitrates had been accumulating here since 1907 and had become so concentrated by this time, July, 1910, that the only place anything would grow was right along the irrigating furrows and even there the grain was very short and thin. Apparently the water in running through these had washed out a little of the nitrate from time to time and so had reduced the salts to a degree of partial tolerance. The soil on top of this mesa, for some reason, is always wet and one not familiar with the topography of the country would be very apt to suggest that the land was seeped by higher irrigation projects. As a matter of fact, this mesa is at least 200 feet higher than the surrounding country and there is no possible chance for seepage in the sense in which the term is ordinarily used. This peculiar condition seems to have resulted from excessive irrigation and a lack of proper drainage. To use a popular expression, the soil has become "water logged." The superabundance of moisture had most certainly favored nitrate production and the agents which were responsible for the coloring matter, for the soil was as black on the surface as crude oil, and as mealy beneath as wood ashes. Before taking a sample, the surface crust and the next two inches were removed and a section, including the fourth to sixth inch inclusive, was obtained. This came from along an irrigating furrow where the barley was making a feeble struggle. In culture, there was almost no surface growth and only a moderate white deposit on the bottom and sides of the flask. A butyric odor was perceptible. I was rather surprised to find that after thirty days there was an increase of 1.68121 m. g. of nitrogen in the culture.

SAMPLE NO. 25.

As a source for the next sample, I selected a truck garden on the outskirts of a mining town. This was thirty-six miles from the nearest case of nitre trouble of which I had knowledge, and so far as I could learn nothing of the sort had ever been observed here either on the soil or the vegetation. The soil chosen was a very light, deep sandy loam which from its proximity to the river, I took to be of alluvial formation. All kinds of vegetables, together with strawberries, were grown here very successfully. The culture produced with this soil gave a heavy gelatinous membrane, light brown in color, and was made up almost entirely of Azotobacter. The increase in nitrogen in thirty days amounted to 5.8842 m. g.

SAMPLE NO. 26.

Sample No. 26 represents the soil of a young orchard in which several of the small trees on the high ground had died in 1910 and others were looking very suspicious. The ground was first broken in the fall of 1908 and set to apples in the spring of 1909. It was irrigated and cultivated thoroughly that season and had been irrigated for the third

time in 1910 when I took my sample Oct. 25. The soil is a clay loam with considerable gravel, in good condition, and so far as I could observe there was no brown color visible although this had been reported as occurring earlier in the season. The land sloped well so there should be ample opportunity for drainage. When introduced into mannite solution, an infusion of this soil produced a heavy, wrinkled surface membrane, with scattering brown patches, the growth being almost exclusively Azotobacter. The increase in nitrogen in this culture was 14.7105 m. g. in thirty days.

SAMPLE NO. 27.

The material for the next study was obtained from an orchard where evidence of nitre was observed for the first time in the summer of 1910. This was an old orchard and a number of the largest trees were very badly injured but not yet dead. There was no indication of the trouble other than the firing of the green leaves. In an adjacent orchard, there had been heavy loss the previous year and all signs pointed to a repetition of the disaster for 1910. The soil was a heavy clay and my sample consisted of the surface two inches taken between two of the affected trees. The culture obtained from the soil infusion of this soil yielded a heavy, wrinkled, surface membrane with isolated brown patches. The increase in nitrogen in the culture in thirty days amounted to 11.3481 m. g.

SAMPLE NO. 28.

The next sample was taken from what had been a young orchard three years previously. It had been given the best of care which may have hastened the appearance of the destroying agents. The tract contained approximately twenty acres sloping gently to the south and west. Some years before it was set to orchard, a reservoir had been built on the northeast corner, the highest point on the place. This was not a success since it was producing a seeped condition in the lower surrounding country and it had to be abandoned. This was about four years before the orchard was planted. The soil is a clayey loam, for the most part, underlaid with a shale. In 1908 the mealy nature of the surface was first observed. At this time Dr. Headden took a sample and states that the conditions did not afford an opportunity for him to judge the color. I have visited this orchard two different times since then and it has so happened each time I have been there that the soil either has been so extremely dry that no color was visible or else it had just been cultivated and all traces on the irrigating furrows had been obliterated. Four acres of the young orchard died in the spring of 1909 and by fall the area involved had nearly doubled. In Oct., 1910, there were scarcely three acres of the original twenty alive. The living trees were all to be found in the five or six rows along the highest side of the tract. When the first injury appeared in 1909, we learned that this same four acres had given trouble in former years, when the land was in alfalfa, so it was to be expected

that this part of the young orchard would be the first to go. As fast as the trees died they were taken up and in 1910 at least twelve acres were sown to corn. Only a very small per cent of this ever reached a height of eighteen inches, and much of it never came through the ground. All over the area involved there are great barren spots, some of them a half acre in extent, where not even a Russian thistle will grow. A two inch surface sample was taken from one of these spots, October 29, 1910, and contrary to expectation my culture developed a yellowish surface membrane with brown patches. Azotobacter was very plentiful. After thirty days the culture showed an increase of 8.6862 m. g. of nitrogen. Inasmuch as all vegetation had refused to grow where the sample was collected, I had rather imagined that the Azotobacter would be killed out as well, and was not anticipating any such active fixation as was secured.

SAMPLES NOS. 29, 30 AND 31.

The next orchard presents, without exception, the most severe case of nitre that has been called to our attention; severe not only in point of destruction, but in rapidity of spread as well. We have seen orchards where the isolated trees and parts of rows were scattered over a large area, but nowhere else have we observed one solid row after another, the entire length of the orchard, go down in rapid succession, as clean as before a forest fire. The original orchard covered about 15 acres of ground sloping gently to the south and, as measured by its producing capacity, was in excellent condition up to the winter of 1909-1910. At this time a spot about twelve feet in diameter appeared at the lower edge of the tract, which the manager stated always looked wet and black in color. Little attention was paid to this until the spring of 1910 when the trees in this vicinity began dying as with nitre. The trouble spread rapidly up hill and back into the orchard so that by the end of the summer 1910 two and one-half acres had been killed and approximately two acres had been taken up.

I visited the ranch on Oct. 29th and found the barren area very wet and boggy in places. The mud was exceedingly sticky and so soft in spots that one would sink down ankle deep in walking over it. Such spots were usually dark brown or black in color and a little higher than the adjacent ground on which a deposit of white alkali had formed following a light snow. I was told that this was the first time the white alkali had been in evidence. The greater part of the barren area was white except for isolated elevated patches and a strip twelve to fifteen feet wide along the upper side which was black. All indications seemed to point to the fact that the portion occupied by the white salts was too wet for the development of the black pigment. That the reader may have some conception of the violence of the attack, I may say that plum trees were pointed out to me which three weeks before were in perfect condition and now were absolutely dead. For four rows back from the edge of the barren area the trees were either

dead or dying (See Fig. 3, Page 23) and beyond these the burning seemed to stop. However, twelve rows farther back on the higher ground I discovered a single tree which was firing. There was a little white alkali nearby but no sign of any brown or black color. I took Sample No. 29 from the surface of the soil near this tree. So far as I could see, this was the only tree in that part of the orchard which was suffering at this time. I visited this same spot January 31, 1911, and the spectacle that greeted me was awful, to express it mildly. Where three months before there was but a single tree affected, there

Fig. 3. Section of an orchard killed by nitre. Photographed October 29, 1910.

were now six to eight acres involved. The soil was brown and very mealy. The orchard manager informed me that most of this change had followed the last irrigation of the orchard which was given about December 1, 1910. He related that three or four days after he had finished irrigating, he noticed a dark brown, oil-like spot ten inches in diameter near the tree from which sample No. 29 was taken and that after twenty-four hours this had increased to twenty-four feet in diameter by actual measurment. In spite of all efforts to break down this testimony by cross examination and conservative suggestions, my informant held firmly to the original statement declaring that there was absolutely no exaggeration. This particular instance most certainly holds all previous records for rapid progress. It was

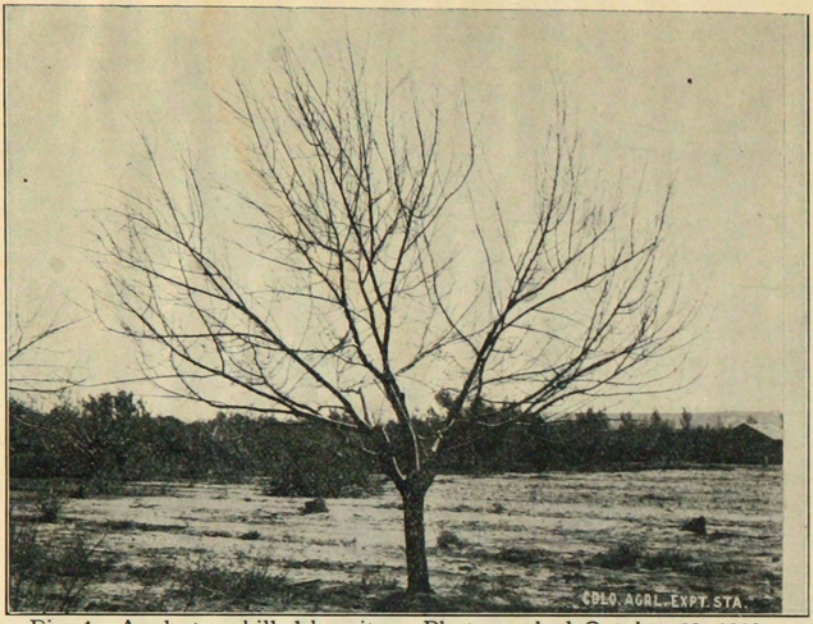

Fig. 4. Apple tree killed by nitre. Photographed October 29, 1910.

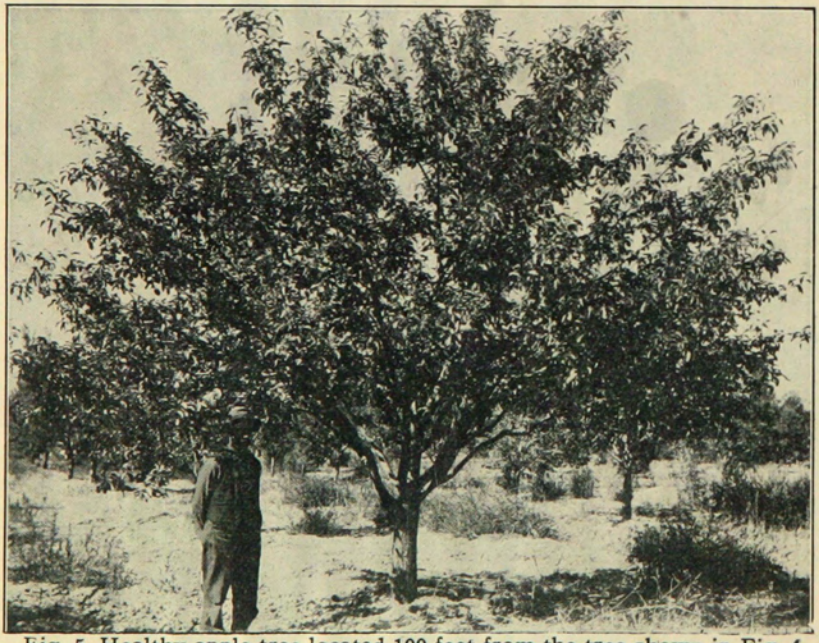

Fig. 5. Healthy apple tree located 100 feet from the tree shown in Fig. 4, and photographed on the same day.

from this spot as a focal center that the malady had spread so as to involve eight acres in an entirely new part of the orchard. On April 18, 1911, I looked over this proposition again. There was not a shadow of a doubt in my mind but that the whole fifteen acres were destined to go sooner or later. A conservative figure of the loss up to this date was thirteen acres and the balance were dying. The property had changed hands three times since my first acquaintance with it and the present owner, believing that drainage would relieve the difficulty, expended over $4000 in putting in some 15,000 feet of drain tile. Some little water flows from this drain but as far as ameliorating the condition of the orchard, there seems to be practically no change. Sample No. 30 was taken from one of the black, wet spots mentioned. Since these were of very recent appearance I was interested in knowing whether this intense black color was necessarily an indication of the concentration of the nitrates. On the one hand, if that was true, I should expect to get only a very slight fixation of nitrogen in the culture; on the other hand, if it bore no immediate relation to excessive quantities of the salt, then I would look for larger results.

Sample No. 31 was collected by Dr. Headden, January 5, 1911, from the eight acre section where the nitrates had developed very rapidly since December, 1909. When he handed me the material, he remarked that he would not be at all surprised if I got no results from this soil for it was as brown and mealy as could be. In culture solution all three of these samples gave typical brown, wrinkled surface membranes which a microscopic examination showed to be rich in Azotobacter. With No. 29, taken from near the single affected tree, the increase in nitrogen in thirty days amounted to 10.2273 m. g.; with No. 30, from the black spot, 10.15725 m. g. and with No. 31, 7.9857 m. g. The result from No. 30 would seem to indicate that the black color does not necessarily mean extremely concentrated nitrates. (Figs. 1, 4 and 5.)

SAMPLE NO. 32.

The next sample was taken October 29, 1910, along the road side near the fence, where the soil looked mealy but where there was no discoloration, which could be accounted for probably by the dry condition of the soil. This spot was selected because the road mentioned ran alongside a young orchard where the trees had been dying from some unknown cause. There was such a combination of factors at work in the orchard, namely, neglect, drought and possibly nitre that it was hardly safe to venture an opinion on the cause of the death of the trees. An alfalfa field adjoining this tract showed many barren spots which were suggestive at least. In culture, this soil developed a heavy, tough, brownish membrane, rich in Azotobacter and after thirty days gave an increase of 15.411 m. g. of nitrogen.

TABLE No. 2. Summary of Nitrogen Fixing Power of Soils, Numbers 1 to 32, in Mannite Solution.

Sample No.	Source	Date of Collection	Date of Examination	Date of Completion	Milligrams Nitrogen at beginning	Milligrams Nitrogen at end	Milligrams Nitrogen fixed per 1.5 grams Mannite, 30 days
1	Orchard—surface crust ¼ inch	Sept. 21, 1909	Oct. 5, 1909	Nov. 4, 1910	4.48320	5.53395	1.05075
2	Orchard—4th to 6th inch	Sept. 21, 1909	Oct. 5, 1909	Nov. 4, 1909	.84060	1.40100	.56040
3	Orchard—12th to 14th inch	Sept. 21, 1909	Oct. 5, 1909	Nov. 4, 1909	.77055	4.2030	3.43245
4	Orchard—surface removed 2nd to 6th inch	Sept. 21, 1909	Oct. 5, 1909	Nov. 4, 1909	1.12080	13.5897	12.46890
5	Orchard—surface removed 2nd to 6th inch	Sept. 21, 1909	Oct. 5, 1909	Nov. 4, 1909	.98070	4.06290	3.08220
6	Beet field—irrigating furrow, 2nd to 6th inch	Sept. 22, 1909	Oct. 5, 1909	Nov. 4, 1909	1.75115	5.3238	3.57265
7	Deserted land—barren spot, 2nd to 6th inch	Sept. 24, 1909	Oct. 5, 1909	Nov. 4, 1909	3.08220	6.09435	3.01215
8	Same as No. 7—outside barren spot, 2nd to 6th inch	Sept. 24, 1909	Oct. 5, 1909	Nov. 4, 1909	3.0822	5.95425	2.87205
9	Alfalfa field—normal, 2nd to 6th inch	Sept. 24, 1909	Oct. 5, 1909	Nov. 4, 1909	1.54110	11.69835	10.15725
10	Orchard—edge of irrigating furrow, surface 2 inches	April 26, 1910	April 27, 1910	June 26, 1910	.9807	8.6862	7.7055
11	Alfalfa field—surface 4 inches	June 24, 1910	June 27, 1910	July 27, 1910	3.43245	8.5461	5.11365

TABLE No. 2 (Continued) Summary of Nitrogen Fixing Power of Soils, Numbers 1 to 32, in Mannite Solution.

Sample No.	Source	Date of Collection	Date of Examination	Date of Completion	Milligrams Nitrogen at beginning	Milligrams Nitrogen at end	Milligrams Nitrogen fixed per 1.5 grams Mannite, 30 days
12	Truck garden—surface 3 inches	July 4, 1910	July 4, 1910	Aug. 3, 1910	5.39385	14.01	8.61615
13	Orchard—surface 3 in. from irrigating furrows	July 13, 1910	July 23, 1910	Aug. 22, 1910	.63045	4.76340	4.13295
14	Orchard—surface 3 in. from irrigating furrows	July 13, 1910	July 23, 1910	Aug. 22, 1910	.07005	6.72480	6.65475
15	Orchard—surface 3 in. from irrigating furrows	July 13, 1910	July 23, 1910	Aug. 22, 1910	.35025	10.50750	10.15725
16	Raw land from adobe hill—surface 3 inches	July 13, 1910	July 23, 1910	Aug. 22, 1910	.00000	.28020	.28020
17	Young orchard on adobe shale—surface 2 inches along ditch.	Oct. 26, 1910	Nov. 9, 1910	Dec. 9, 1910	1.33095	.7005	0.0000
18	Same as No. 17—surface 2 inches between trees	Oct. 26, 1910	Nov. 9, 1910	Dec. 9, 1910	.49035	.8406	.35025
19	Orchard—surface 3 in. between irrigating furrows	July 1, 1910	July 23, 1910	Aug. 22, 1910	.4203	10.22730	9.80700
20	Orchard—surface 3 in. between trees	July 16, 1910	July 23, 1910	Aug. 22, 1910	.76050	3.22230	2.52180
21	Orchard—surface 2 in. from irrigating furrow	July 14, 1910	July 23, 1910	Aug. 22, 1910	.28080	3.08220	2.80140

TABLE No. 2, (Continued). Summary of Nitrogen Fixing Power of Soils, Numbers 1 to 32, in Mannite Solution.

Sample No.	Source	Date of Collection	Date of Examination	Date of Completion	Milligrams Nitrogen at beginning	Milligrams Nitrogen at end	Milligrams Nitrogen fixed per 1.5 grams Mannite, 30 days
22	Orchard—surface 2 in. from irrigating furrow	July 14, 1910	July 23, 1910	Aug. 22, 1910	.21015	9.10650	8.89635
23	Orchard—surface 3 in. between irrigating furrows	July 16, 1910	July 23, 1910	Aug. 22, 1910	.35025	7.49535	7.14510
24	Barley field—black surface 3 inches near irrigating furrow	July 20, 1910	July 23, 1910	Aug. 22, 1910	.35025	2.03146	1.68121
25	Truck patch—normal, surface 3 inches	July 21, 1910	July 23, 1910	Aug. 22, 1910	.42030	6.30450	5.88420
26	Young orchard—surface 3 inches	Oct. 26, 1910	Nov. 9, 1910	Dec. 9, 1910	.2802	14.9907	14.7105
27	Orchard—surface 3 inches	Oct. 26, 1910	Nov. 9, 1910	Dec. 9, 1910	.4203	11.7684	11.3481
28	Young Orchard—surface 3 inches	Oct. 26, 1910	Nov. 2, 1910	Dec. 2, 1910	.35025	9.03645	8.68620
29	Orchard—surface 2 inches	Oct. 29, 1910	Nov. 2, 1910	Dec. 2, 1910	.4203	10.6476	10.2273
30	Same as No. 29—surface inch of black spot	Oct. 29, 1910	Nov. 2, 1910	Dec. 2, 1910	3.29235	13.4496	10.15725
31	Same as No. 29—mealy surface 3 inches	Jan. 5, 1911	Jan. 6, 1911	Feb. 5, 1911	.49035	8.47605	7.98570
32	Orchard roadside—mealy soil under dust.	Oct. 29, 1910	Nov. 2, 1910	Dec. 2, 1910	.4203	15.8313	15.4110

While it may be early to state any conclusions, even tentatively held, the foregoing work suggests the following:

1. Excessively high nitrates in the soil will kill the Azotobacter flora.
2. A limited amount of soil nitrate does not seriously affect the nitrogen fixing power of a soil.
3. Our adobe shale soils, both in the raw state and when newly cultivated, possess little if any nitrogen fixing power.
4. The nitrogen fixing power of our soils is not limited to any geographical locality or class of soils, however, the degree of activity may vary.
5. The power to fix atmospheric nitrogen is a property common to many cultivated Colorado soils.
6. Azotobacter chroococcum appears to be the dominant nitrogen fixing agent.

THE NITROGEN FIXING POWER OF SOILS IN SITU.

Having satisfied ourselves that certain Colorado soils possessed the power of fixing atmospheric nitrogen in solutions, the next point on which we wished to inform ourselves was whether these same soils had the power of fixing nitrogen *in situ*. If that could be demonstrated, it would be a relatively simple matter to explain the high nitrates, for, given the proteid nitrogen from which to make the nitrates, we felt reasonably certain that the ammonifying and nitrifying flora would take care of the conversion.

For this part of the investigation two samples of soil were selected at random, one from the central part of the state, and the other from the northern. Both were from localities where either the nitre trouble or the brown stain had been observed. The nitrogen fixing power of these soils was determined independently by two different workers, the northern Colorado sample by Dr. Headden, and the central by the writer. The soils were not handled in the same way by the two of us, so it may be well to discuss our respective manipulations. Other soils have been studied but these two are of particular interest since the results were obtained independently in different laboratories.

Northern Sample.

This soil was collected by Dr. Headden, December 12, 1910, from the young orchard designated as No. 10 in the preceding series. It was screened in a moist condition through a twenty-five mesh wire screen and 1200 grams of the moist soil, with no further treatment, were placed in a deep culture basin (10 in. x 2 in.) and pressed down firmly. The soil moisture was determined and sufficient boiled distilled water was added to make eighteen to twenty percent moisture. This was maintained throughout the experiment. The soil was incubated for twenty-seven days in the dark at 28°C. to 30°C. at the end

of which time samples were removed for analysis and the total nitrogen determined. This showed a gain of 10.54 m. g. of nitrogen per 100 grams of soil for the 27 days.

I am indebted to Dr. Headden for the following results:

Total nitrogen at the end of 27 days - 117.79 m. g. per 100 g. soil.
Total nitrogen at the beginning, - 107.25 m. g. per 100 g. soil.
Total nitrogen gained by fixation in 27 days, ---------- 10.54 m. g. per 100 g. soil.

Assuming that the fixation, under field conditions, proceeds uniformly at this same rate for six months, it would mean an addition of 475.26 pounds of nitrogen or 2,970.41 pounds of protein per month for every acre foot, and in six months, this would amount to 2,851.60 pounds of nitrogen or 17,822.50 pounds of protein, while in a year, we should have 5,703.20 pounds of nitrogen or 35,645.00 pounds of protein per acre foot. With this increase of 2.85 tons of nitrogen or 17.82 tons of protein per acre foot in one year, there certainly need be no cause for anxiety over a source of nitrogen for high nitrate formation.

Central Sample.

This soil was collected by the writer December 30, 1910, and was from the same source as the sample designated as No. 29 of the preceding series. The soil was first air dried and then passed through a 40 mesh wire screen. The moisture was determined and found to be 2.1 per cent. Sufficient sterile distilled water was next placed in a deep culture dish (100 m. m. x 30 m. m.) to give 100 grams of the air dried soil a water content of 10 per cent. 100 grams of the soil were next added to the water in the culture dish and the weight of the whole was determined. This weight was kept constant by daily additions of sterile distilled water throughout the experimental period. The soil was incubated in the dark at 28°C. to 30°C. for thirty days at the end of which time samples were removed and the total nitrogen determined. For every 100 grams of soil there was an increase of 8.22 m. g. of nitrogen in thirty days.

Total nitrogen at the end of 30 days, - - 82.11 m. g. per 100 g. soil
Total nitrogen at the beginning, - - - 73.89 m. g. per 100 g. soil
Total nitrogen gained by fixation in 30 days, 8.22 m. g. per 100 g. soil

Considering that the fixation, under field conditions, proceeds uniformaly at this same rate for six months, it would mean an additon of 333.60 pounds of nitrogen or 2,085.00 pounds of protein per month for each acre foot, or in six months this would amount to 2,001.60 pounds of nitrogen or 12,510.00 pounds of protein, while in one year we should have 4,003.2 pounds of nitrogen or 25,020.00 pounds of protein. Expressing this in tons per acre-foot per annum, we get an increase of 2.001 tons of nitrogen or 12.5 tons of protein.

Table No. 3. Summary of Fixation of Nitrogen in Soils in Situ.

Source of Sample	Duration of Experiment	Milligrams nitrogen per 100 g. soil at beginning.	Milligrams nitrogen per 100 g. soil at end.	Milligrams nitrogen fixed per 100 g. soil
Northern Colorado	27 days	107.25	117.79	10.54
Central Colorado	30 days	73.89	82.11	8.22

Table No. 4. Increase in Nitrogen per 100 g. of Soil.

Source of sample	Increase in nitrogen per 100 g. soil as					
	Milligrams nitrogen		Milligrams protein		Milligrams $NaNO_3$	
	1 month	1 year	1 month	1 year	1 month	1 year
Northern Colorado	11.88	142.58	74.26	891.12	72.08	865.14
Central Colorado	8.34	100.08	52.12	625.50	50.60	607.26

Table No. 5. Increase in Nitrogen per Acre-foot of Soil.

Source of sample	Increase in nitrogen per acre-foot of soil as					
	Pounds nitrogen		Pounds protein		Pounds $NaNO_3$	
	1 month	1 year	1 month	1 year	1 month	1 year
Northern Colorado	475.26	5,703.20	2,970.41	35,645.00	2,883.82	34,605.87
Central Colorado	333.60	4,003.2	2,085.00	25,020.00	2,024.21	24,290.61

Relation of Soil Moisture to Fixation of Nitrogen in Soil.

It has been a matter of frequent observation that where there is an excess of water, as in land which is unquestionably seeped, or where there is a liberal coating of white alkali on the surface, a condition indicative of poor drainage, neither the brown color nor the high nitrates are to be found. However, along the margin of such areas on the higher ground where there is an abundant supply of moisture but not too much, we are apt to find both the high nitrates and the brown color. These two conditions, we have come to associate with the presence of the nitrogen fixing organisms in the soil, and the resulting nitrogen fixing power of that soil. We do not mean to say that they are, by any means, a necessary accompaniment, yet they are very often found together, and from certain experiments which we have made, directed especially toward this feature of the problem, we are led to believe that the relation existing among these three factors is a dependent one.

One step toward the proof of this lay in the demonstration of the relation of the moisture content of the soil to the fixation of nitrogen. To this end, six deep culture dishes were prepared with varying amounts of sterile distilled water each containing sufficient to give 100 grams of air dried soil with a moisture content of 2.1 per cent the following degrees of moisture: 2.1, 10, 20, 25, 30, and 48 per cent respectively. 100 grams of air dried soil which was known to possess nitrogen fixing powers *in situ* were added to each dish. It will be noted that the first dish contained the air dried soil only, while the last, with 48 per cent water, was saturated. The weight of each dish and its contents was determined, and every day the loss of water by evaporation was restored with sterile distilled water. The soils were all kept in the incubator at a temperature of 28°C. to 30°C. for thirty days at the end of which time the total nitrogen was determined for each. The results of the experiment are given in Table No. 6.

TABLE No. 6. Relation of Soil Moisture to Nitrogen Fixation in Soil.

Per cent moisture	Milligrams nitrogen per 100 g. soil		Milligrams nitrogen fixed per 100 g. soil in 30 days
	At beginning	After 30 days	
2.1	73.89	78.84	4.95
10.0	73.89	82.11	8.22
20.0	73.89	80.90	7.01
25.0	73.89	79.13	5.24
30.0	73.89	78.49	4.60
48.0	73.89	73.85	....

The experiment indicates that the optimum moisture content for maximum fixation lies between 10 per cent and 20 per cent; that the amount of fixation gradually decreases as the saturation point of the soil is approached at which it is zero. These results are in perfect harmony with our field observations which have pointed clearly to the detrimental effect of excessive moisture both on the production of brown color and the formation of high nitrates.

THE RELATION OF NITRATES AND AZOTOBACTER CHROOCOCCUM TO THE BROWN COLOR.

The continual occurrence of the brown color on high nitre soils, which have been shown to possess nitrogen fixing power, is too constant an association to be regarded as a mere accident or coincidence. This relation needs no further exposition since it has been referred to repeatedly in the preceding pages, but before entering into any discussion of the subject, it should be understood clearly and emphatically that we have no intention of appealing to the nitrates or the nitrogen fixing flora of the soil to explain *every* brown spot or similar discoloration that may be found. There are at least two other recognized agents that may be responsible for a similar condition. I refer to the

well known Black Alkali of the Southwest in which sodium carbonate is the active principle in bringing the soil humus into solution, which solution, being highly colored, may give the surface a dark appearance. Again there are some soils which contain sufficient quantities of calcium chloride to absorb enough moisture to impart a dark color to the soil. I have Dr. Headden's statement that none of the soils which are concerned in this project contain enough of either sodium carbonate or calcium chloride to account for this phenomenon. Our problem is manifestly different from either of these.

In our pure culture studies of the Azotobacter flora of these soils, we have isolated what appear, in the final analysis, to be six or seven varieties of Azotobacter chroococcum. Three of these, at one time or another, have produced the characteristic brown color on mannite agar. One of these, No. 3, has maintained this character undiminished since it was first isolated; the second, No. 93, acquired the color three weeks after isolation, retained it for six weeks and then lost it; the third, No. 1, has produced a small amount of a light brown pigment at times ever since its isolation but there has been nothing constant in this respect until the last six weeks when it has begun to produce a heavy dark brown color. Three of the remaining four cultures, Nos. 4, 8 and 10, have been characterized by their spreading nature and their abundant, moist, raised, gelatinous, starchy white to yellowish growth on mannite agar. Morphologically and culturally, these three possess such differences as seem to make them distinct from one another; the fourth, No. 13, differed from all the rest in the production of a delicate cream colored pigment; it was spreading in habit but flat, not gelatinous and grew only moderately well on mannite agar. Unfortunately, this culture was lost early in our work, and consequently it has been given no consideration in this treatise beyond mere mention.

The brown color of cultures 1, 3, and 93 served to identify them beyond reasonable doubt as Azotobacter chroococcum. The other four cultures were left unclassified, for the time being, except to place them in the genus Azotobacter after they had been shown to possess nitrogen fixing powers in pure culture.

The close resemblance between the brown pigment formed by some of our cultures and the brown color present on certain soils was suggestive to say the least. It seemed reasonable to me that there might be something peculiar to our soils which could stimulate and intensify the pigment producing power of Azotobacter chroococcum.

To determine this, a number of synthetic agars were prepared, the composition of which was based upon the water soluble salts present in a certain nitre soil. The carbon was supplied in the form of mannite. Each agar differed from every other in the omission of one of the compounds, our object being to determine by elimination, if possible, if any one constituent was directly responsible for the brown pigment. The analysis of the water soluble salts in the soil which was

used as a basis for preparing the different agars, together with the composition of the various solutions from which the agars were made is given below. The solid medium was prepared by adding 15 grams of agar to each 1000 c. c. of solution.

**Water soluble salts in soil used as a basis for synthetic agars.*

	Per Cent
$CaSO_4$	15.902
$MgSO_4$	2.942
K_2SO_4	3.387
Na_2SO_4	15.264
Na_2CO_3	4.813
Na Cl	34.145
$NaNO_3$	22.781
Silicic Acid	.252
Loss (water, organic matter, etc.)	.471

The water soluble amounted to 2.97 per cent of the air dried material.

Solution lacking Calcium Sulphate ($CaSO_4$)

Distilled water	1000.00	c. c.
Na_2SO_4	9.0668	grams
Na_2CO_3	2.8589	grams
Na Cl	20.2621	grams
$NaNO_3$	13.5319	grams
K_2SO_4	2.0118	grams
$MgSO_4$	1.7475	grams
Mannite	15.0000	grams

Solution lacking Sodium Carbonate (Na_2CO_3)

Distilled water	1000.00	c. c.
$CaSO_4$	9.4457	grams
Na_2SO_4	9.0668	grams
Na Cl	20.2621	grams
$NaNO_3$	13.5319	grams
K_2SO_4	2.0118	grams
$MgSO_4$	1.7475	grams
Mannite	15.0000	grams

Solution lacking Sodium Chloride ($NaCl$)

Distilled water	1000.00	c. c.
$CaSO_4$	9.4457	grams
Na_2SO_4	9.0668	grams
Na_2CO_3	2.8589	grams
$NaNO_3$	13.5319	grams
K_2SO_4	2.0118	grams
$MgSO_4$	1.7475	grams
Mannite	15.0000	grams

*Furnished by Dr. Headden. Bul. 155, p. 17, Analysis XV, Colo. Exp. Sta.

Solution lacking Sodium Nitrate ($Na\ No_3$.)

Distilled water	1000.00	c. c.
$Ca\ SO_4$	9.4457	grams
$Na_2\ SO_4$	9.0668	grams
$Na_2\ CO_3$	2.8589	grams
Na Cl	20.2621	grams
$K_2\ SO_4$	2.0118	grams
$Mg\ SO_4$	1.7475	grams
Mannite	15.0000	grams

Solution lacking Sodium Sulphate ($Na_2\ SO_4$)

Distilled water	1000.00	c. c.
$Ca\ SO_4$	9.4457	grams
$Na_2\ CO_3$	2.8589	grams
Na Cl	20.2621	grams
$Na\ NO_3$	13.5319	grams
$K_2\ SO_4$	2.0118	grams
$Mg\ SO_4$	1.7475	grams
Mannite	15.0000	grams

Solution lacking Magnesium Sulphate ($Mg\ SO_4$)

Distilled water	1000.00	c. c.
$Ca\ SO_4$	9.4457	grams
$Na_2\ SO_4$	9.0668	grams
$Na_2\ CO_3$	2.8589	grams
Na Cl	20.2621	grams
$Na\ NO_3$	13.5319	grams
$K_2\ SO_4$	2.0118	grams
Mannite	15.0000	grams

Solution lacking Potassium Sulphate ($K_2\ SO_4$)

Distilled water	1000.00	c. c.
$Ca\ SO_4$	9.4457	grams
$Na_2\ SO_4$	9.0668	grams
$Na_2\ CO_3$	2.8589	grams
Na Cl	20.2621	grams
$Na\ NO_3$	13.5319	grams
$Mg\ SO_4$	1.7475	grams
Mannite	15.0000	grams

Solution lacking Mannite ($C_6H_{14}O_6$)

Distilled water	1000.00	c. c.
$Ca\ SO_4$	9.4457	grams
$Na_2\ SO_4$	9.0668	grams
$Na_2\ CO_3$	2.8589	grams
Na Cl	20.2621	grams
$Na\ NO_3$	13.5319	grams
$K_2\ SO_4$	2.0118	grams
$Mg\ SO_4$	1.7475	grams

The chemical reaction of these solutions was left unchanged. Agars of four different strengths were prepared from each solution by diluting the solution once, twice and three times with an equal volume of distilled water. The resulting agars, then, contained the

Table No. 7. Growth and Pigment Production on Synthetic Soil Agars.

Culture	Synthetic Agar lacking							
	$CaSO_4$		Na_2SO_4		Na_2CO_3		NaCl	
	Growth	Pigment	Growth	Pigment	Growth	Pigment	Growth	Pigment
No. 3.	moderate	brown	moderate	brown in spots	moderate	chocolate brown	moderate	brown
No. 8.	moderate	black	moderate	deep chocolate brown	moderate	black	moderate	chocolate brown
No. 93	moderate	chocolate brown	moderate	chocolate to black	moderate	chocolate to black.	moderate	chocolate brown
A. chroococcum.	moderate	dark brown to black	moderate	dark brown to chocolate	slight	none	slight	light brown

Culture	Synthetic Agar lacking							
	$NaNO_3$		K_2SO_4		$MgSO_4$		Mannite	
	Growth	Pigment	Growth	Pigment	Growth	Pigment	Growth	Pigment
No. 3	slight	none	moderate	chocolate brown	moderate	chocolate brown	very slight	none
No. 8	moderate	few dark brown specks	moderate	black	moderate	black	very slight	none
No. 93	moderate	few brown specks at bottom of streak	moderate	dark brown	moderate	black	very slight	none
A. chroococcum.	slight	none	moderate	dark brown	moderate	dark brown	very slight	none

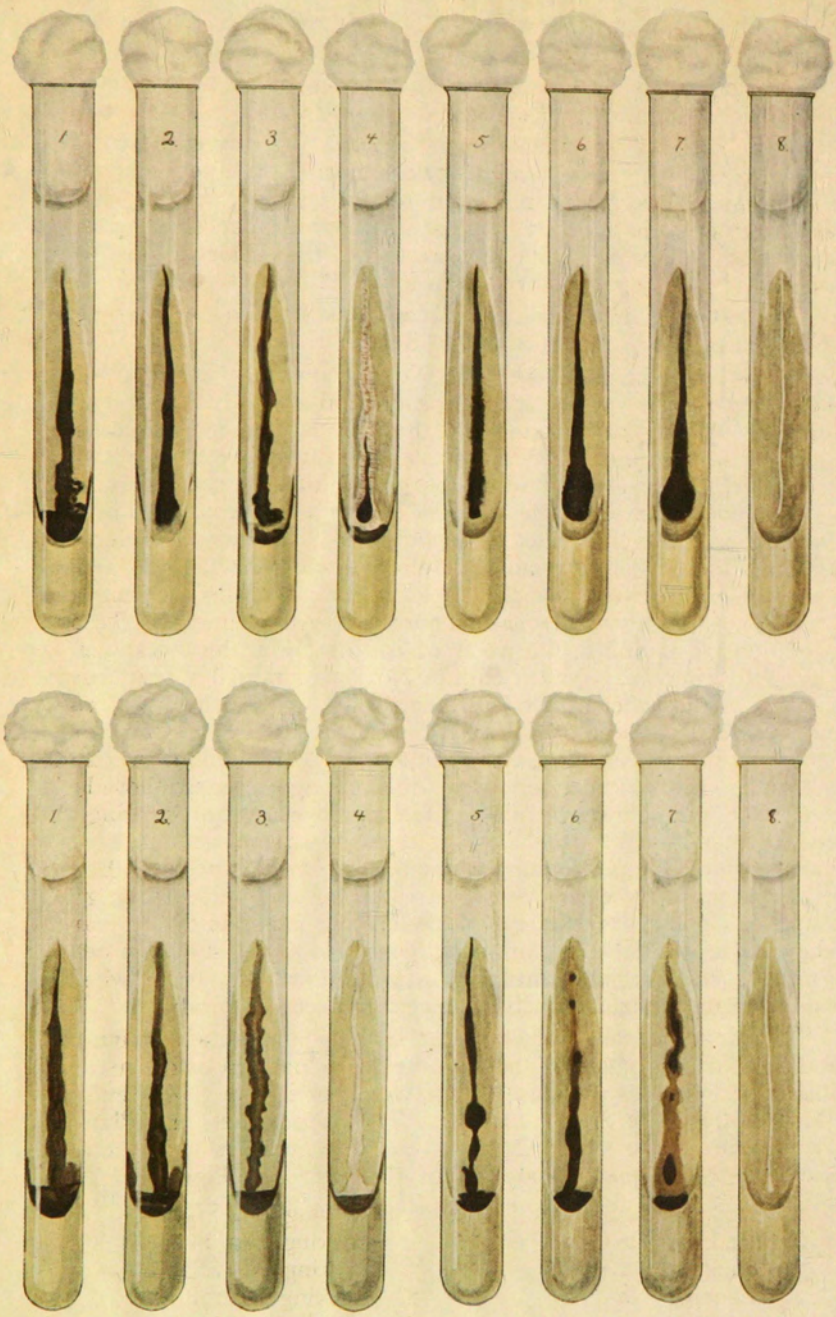

PLATE I

COLO. AGRL. EXPT. STA.

above solutions in the following amounts in which S represents the original strength: S, S÷2, S÷4, S÷8.

The agar was placed in test tubes, using about 7 c.c. per tube, and sterilized for 5 minutes in the autoclave at 120°C.

Streak cultures were next made on each agar of each strength, using cultures Nos. 3, 8, 93 and a stock culture of A. chroococcum. It will be remembered that No. 3 had retained its pigment producing power since isolation, No. 93 had lost it and No. 8 had shown little if any color beyond a dirty white. The results of these inoculations, when observed after fifteen days, are given in Table No. 7.

The best growth as well as pigment was secured on those agars represented by the formulae S÷4 and S÷8.

An inspection of the above table, No. 7, shows very clearly that the two limiting factors in the pigment production are sodium nitrate and mannite. The growth obtained on the agar lacking mannite was so very slight that it was, indeed, difficult to say whether there was any actual growth or whether it was just the line of the original transfer. This was not the case in the agar lacking sodium nitrate. The line of inoculation was well defined in all and in two there was a moderately heavy growth. There was absolutely no brown color but only a dirty white with culture No. 3 and the stock culture of A. chroococcum. In culture No. 8, there was a small amount of brown pigment at the bottom of the streak, and in the water of condensation, the remainder of the growth being dirty white; culture No. 93 contained a few brownish specks in the water of condensation, which under ordinary circumstances would have been overlooked; the streak proper was dirty white in color. Without exception, all the cultures produced abundant chocolate brown to black pigment on all the different agars except those lacking mannite and sodium nitrate. I feel that we are not begging the question when I make the statement that the reason we obtained no pigment in the absence of mannite was because we had no growth. To me, it was perfectly clear from the results of this series, that given a source of energy, the nitrate was the limiting factor in the formation of the dark brown color. I am not prepared to say, just now, whether the nitrate acts as a stimulant to growth, pure and simple, or whether it exercises an oxidizing function on certain bacterial products.

The results of this study were so striking and so self convincing that I have had two sets of the cultures reproduced in colors as nearly like the originals as possible. These were made from 20 day cultures by Miss Palmer, the Station artist. The upper set in Plate I, shows Culture 8, the lower, Culture 93. The numbers 1, 2, 3, etc. on the tubes indicate the composition of the agar as follows:

1. Lacking $Ca\,SO_4$
2. Lacking $Na_2\,CO_3$
3. Lacking $Na\,Cl$
4. Lacking $Na\,NO_3$
5. Lacking $Na_2\,SO_4$
6. Lacking $Mg\,SO_4$
7. Lacking $K_2\,SO_4$
8. Lacking Mannite.

By a system of elimination, we have shown above, that in the absence of nitrates there is practically no pigment formation. I was interested next in knowing just how necessary the other salts were to the production of the brown color, and whether the nitrates alone might not give the desired result. In order to determine this last point, a stock glucose agar was prepared as follows:

Stock Glucose Agar.

Tap water	1000 c. c.
Glucose	20 grams
Agar-agar	20 grams

Glucose was substituted for mannite since two of my cultures produced pigment on the standard mannite agar, and it was determined experimentally that if this substitution was made in the stock agar, practically no color resulted with any of the cultures. By doing this all brown pigment producing factors were eliminated, and I had a medium which would support growth and to which the limiting compounds could be added.

A 10 per cent solution of $NaNO_3$ was prepared in distilled water and sufficient quantities of this were added to different lots of the stock glucose agar to give them a $NaNO_3$ content of 0.0, .01, .03, .05, .08, 0.1, 0.3 and 0.5 per cent respectively. In a 10 per cent solution of $NaNO_3$, 0.1 c. c. contains .01 grams of $NaNO_3$. In order to obtain the above percentages, the following amounts of this 10 per cent solution were added to respective 50 c. c. lots of liquified stock glucose agar: 0.0, .05 c. c., 0.15 c.c., 0.25 c.c., 0.4 c.c., 0.5 c.c., 1.5 c.c., and 2.5 c.c. The agar was placed in test tubes, sterilized for five minutes at 120°C. in the autoclave and slanted. Agar stroke inoculations were made on these with cultures Nos. 1, 3, 4, 8, 10, 93 and stock A. chroococcum

Our results with the series were gratifying beyond expectation. At the end of fourteen days, we had secured either an intense chocolate brown or a black pigment with every one of our cultures on those agars which contained the $NaNO_3$, but absolutely none on the control. The pigment varied in intensity with the amount of $NaNO_3$ present, the optimum quantity for the darkest pigment being between .05 and .08 per cent. In the early growth of the cultures a very nice gradation could be seen in the intensity of the colors, beginning with none in the control, a light brown in the .01 per cent, and a shade darker in each tube as the amount of $NaNO_3$ increased until the deep chocolate brown or black was reached at .05 and .08 per cent after which the shade of brown became somewhat lighter and remained almost constant. With age, this gradation of color was lost, all tubes except .01 and .03 per cent, showing an almost uniformly dark chocolate brown or black pigment. Plate II, prepared from twenty day cultures, illustrates culture No. 8 of this series. Beginning at the left hand side, the tubes contain 0.0, .01, .03, .05, .08, 0.1, 0.3 and 0.5 per cent of

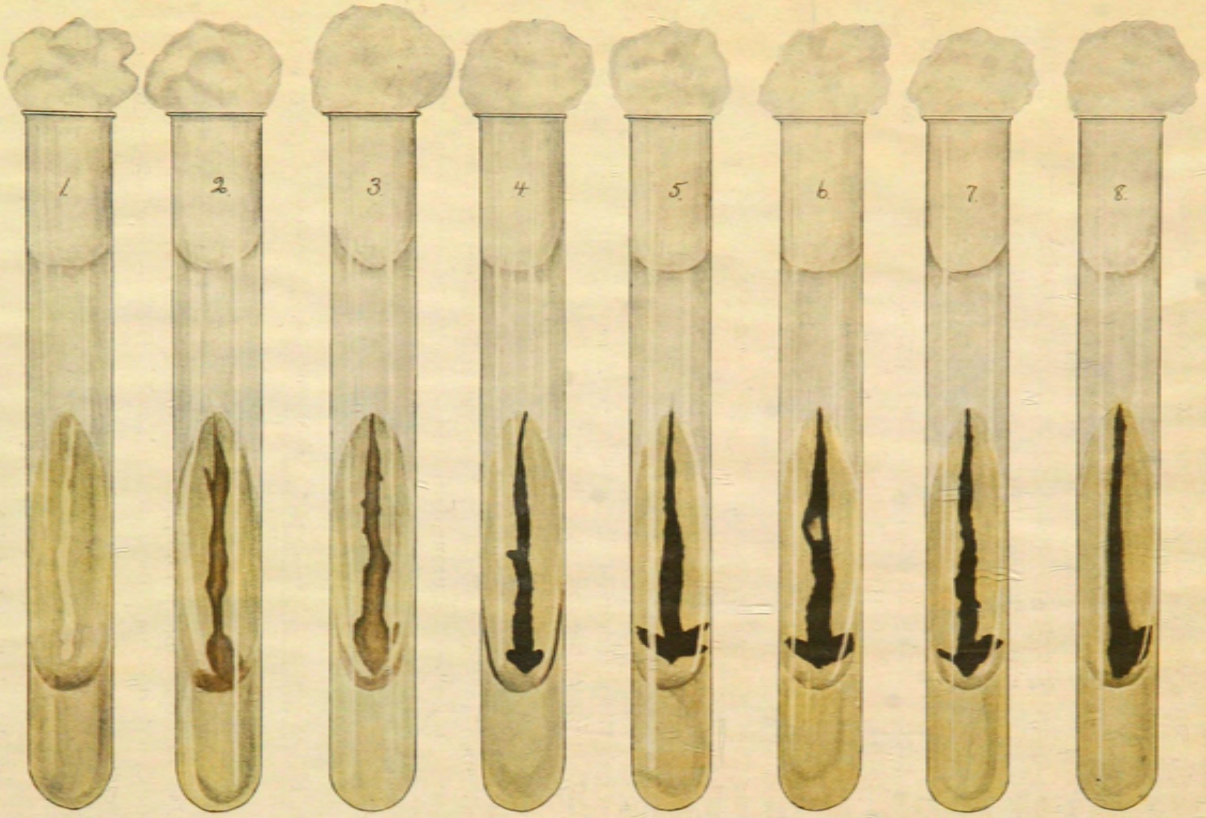

PLATE II

COLO. AGRL. EXPT. STA.

$Na\ NO_3$ respectively. After one has seen this experiment there can be no questioning the fact, that given a supply of carbon, $Na\ NO_3$, of and by itself, can cause A. chroococcum to produce a chocolate brown to black pigment.

This observation is substantiated by the work of Beijerinck[1] in which he has shown that "Pigment formation could also be observed in pure cultures if the mannite was replaced by dextrose and *nitrate* in minimum quantities was added." In the application of these results to field conditions, we have a very tenable explanation of the brown color of the soil. It has been shown that these soils are abundantly stocked with Azotobacter chroococcum, and in the presence of the large amount of nitrates which they carry, the inevitable consequence must be the production of an intensely brown pigment, which has brought the brown spots to our attention.

In 1904 Heinze[2] expressed the view that possibly the dark color of soil was due in a degree to the pigment of A. chroococcum. Lohnis[3] was not inclined to accept this statement, but Omeliansky and Ssewerowa[4] are of the opinion that while it would be a mistake to attribute the dark color of soils to this cause altogether, one has no right to deny the possibility of its occurrence. They have shown experimentally that a brown pigment is produced by Azotobacter in a medium containing chalk and hydrolized starch, both of which are present in soils as $Ca\ CO_3$ and as decomposed plant tissue respectively. Therefore, they concluded that, "The part which Azotobacter plays in the dark color of the soil is not to be overlooked."

The intensely brown pigment which all of our cultures have shown in this and the preceding series seems to identify them all as varieties of A. chroococcum, and consequently they may be considered as such in this bulletin. The variation which has been noted before is in perfect harmony with the observations of Omeliansky and Ssewerowa[5] who state that, "Between the colored and colorless, intermediate forms exist in which the pigment formation is more or less limited."

THE RELATION OF NITROGEN COMPOUNDS OTHER THAN NITRATES TO THE PRODUCTION OF BROWN PIGMENT.

Now if nitrates by themselves can bring about pigment production, may not the same be equally true of other forms of nitrogen? To answer this question, a number of different agars were prepared, each containing a different form of nitrogen. The list included nitrogen as peptone, asparagin, ammonium chloride, ammonium sulphate and

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1. Cent. f. Bakt. Abt. II, Bd. 7, p. 561. 1901.
2. Cent. f. Bakt., Abt. II., Bd. 12, p. 357; Bd. 16, p. 341. 1906
3. Lohnis, Handb. d. Landw. Bakt. p. 712.
4. Cent. f. Bakt., Abt. II., Bd. 29 p. p. 649, 650. 1911.
5. Cent. f. Bakt., Abt. 11., Bd. 29, p. 643. 1911.

sodium nitrite. With the exception of the peptone, a solution of each was prepared containing the nitrogen equivalent of a ten per cent. solution of $NaNO_3$. This was done so that the various agars would be comparable to the sodium nitrate series in point of nitrogen content. The percentage equivalents for the salts mentioned, corresponding to a 10 per cent solution of $NaNO_3$ are as follows:

Asparagin	8.8231 per cent
NH_4Cl	6.2887 per cent
$(NH_4)_2SO_4$	6.5894 per cent
$NaNO_2$	8.1189 per cent

The proteid (peptone) agar was made by adding to the stock glucose agar, standard nutrient broth, neutral in reaction, in the following proportions: 0.0, .1, .2, .5, .8, 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, and 10.0 per cent. The other agars were prepared by adding to respective lots of stock glucose agar the above solutions in amounts corresponding to 0.0, .01, .03, .05, .08, 0.1, 0.3 and 0.5 per cent of $NaNO_3$. For every 50 c. c. of the stock glucose agar the following quantities of these solutions were required to give the above percentages: 0.0 c. c., .05 c. c., .15 c. c., .25 c. c., .4 c. c., .5 c. c., 1.5 c. c. and 2.5 c. c. respectively. The six different agars were placed in test tubes using about 7 c. c. each, sterilized in the autoclave for five minutes at 120°C. and slanted. Stroke cultures were made on these employing cultures Nos. 3, 8, 93 and our stock culture of A. chroococcum.

At the end of eighteen days, there was no brown pigment produced by any of the cultures on the proteid nitrogen agar containing beef broth, although there was luxuriant growth in all of the tubes. No pigment whatever, was made by any of the cultures on either the amido nitrogen agar, containing asparagin, or the ammonia nitrogen agars, containing respectively NH_4Cl and $(NH_4)_2SO_4$. The growth ranged from slight to moderate. On the nitrite nitrogen agar, with cultures Nos. 8 and 93, we obtained a decided chocolate brown in the tubes which contained the $NaNO_2$ corresponding to .01 per cent $NaNO_3$. All of the inoculations with these two organisms grew, but without color. Culture No. 3 gave a brown and chocolate brown pigment with $NaNO_2$ corresponding to .01 and .03 per cent $NaNO_3$ respectively. The stock culture of A. chroococcum grew very feebly on this agar as on the others, and produced no pigment. Control cultures on the stock glucose agar, to which no nitrogen was added, were carried along with the above. Growth took place but there was no evidence of any pigmentation.

The results of this work indicate that in the presence of nitrates, A. chroococcum produces an intensely brown to black pigment; that nitrites in certain proportions, exercise this influence to a less degree; and that nitrogen as NH_4Cl, $(NH_4)_2SO_4$, asparagin, and peptone has no effect upon this function.

SOLUBILITY OF THE PIGMENT.

In their extended studies on the formation of pigment by A. chroococcum, Beijerinck[1], Omeliansky and Ssewerowa[2] have found that the pigment is insoluble in the ordinary solvents. On this point, Beijerinck says, "Insoluble in water, alcohol, ether, chloroform, carbon disulphide, the pigment went into solution under the influence of alkalies, whereby it probably underwent a chemical change." Omeliansky and Ssewerowa state that, "The pigment is insoluble in the usual solvents. Only under the action of alkalies does it go into solution, thereby, nevertheless, changing itself chemically."

The relation of alkalies to the solution of pigment, as described by these investigators, suggests a further explanation for the brown stain which we find on the ditch banks and irrigation furrows. May it not be possible that under the influence of the soil nitrates, Azotobacter chroococcum produces an intense pigment which is brought into solution by the alkaline soil waters, and once in solution, the coloring matter is carried to the surface where it becomes concentrated and produces the characteristic appearance? While we have given but little consideration to this explanation of the color, we have reasons for believing that there is more to this hypothesis than mere speculation and idle fancy.

ACKNOWLEDGMENTS.

I wish to acknowledge, with thanks, my indebtedness to Dr. M. W. Beijerinck of Delft, Holland, for the stock cultures of A. chroococcum, A. agilis and A. lactose, which he has so kindly sent me. To Dr. Headden, I am indebted for the problem itself as well as for many field notes and chemical data. To Professor Gillette, the Director, I wish to express my appreciation of the two colored plates, and Miss Palmer, I wish to thank for preparing the originals from which these were made.

SUMMARY.

The power to fix atmospheric nitrogen is a property common to many cultivated Colorado soils.

This power is not confined to nitrogen fixation in solutions, but is manifested in soils as well.

"The rate of fixation of nitrogen obtained is sufficient to account for the nitrates found in the soil provided that it is nitrified. The rate of nitrification obtained is sufficient to account for the formation of the nitrates found, in most cases if not all of them."[3]

The nitrogen fixing power is not limited to any geographical locality or class of soils, however, the adobe shale soils, both in a raw

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[1]Loc. cit., page 39.
[2]Loc. cit., page 39.
[3]Bulletin 178, p. 96, Colo. Exp. Station, 1911.

state and newly cultivated, possess little, if any, nitrogen fixing power.

Excessive nitrates either destroy or greatly attenuate the nitrogen fixing flora of a soil.

A limited amount of soil nitrate does not seriously affect the nitrogen fixing power of a soil.

Azotobacter chroococcum appears to be the dominant nitrogen fixing organism in the soils studied.

The dark brown color of the nitre soils is due, in a large part, to the pigment produced by Azotobacter chroococcum.

Given a source of energy, the nitrate is the limiting factor in the production of the brown color.

In the presence of nitrates, Azotobacter chroococcum develops a chocolate brown to black pigment; nitrites, in certain amounts, produce similar results, but to a less degree; nitrogen as NH_4Cl, $(NH_4)_2SO_4$, asparagin, and peptone has no effect upon this function.

The highly colored extracts obtained from certain nitre soils suggest that the pigment of Azotobacter chroococcum may be soluble in the alkaline soil waters.

Excessive soil moisture, by interfering with the growth of Azotobacter chroococcum, prevents the formation of the brown color on the soil, and makes the fixation of atmospheric nitrogen impossible.