Investigation on the Influence of Aerial-Earth Circuit on the Biological Activities.

I. Influence on Azotobacter chroococcum.[1]

II. Mechanism of the Influence on Azotobacter chroococcum as to its Potential.[2]

By

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The influence of aerial-earth circuit on the biological activities was investigated, and as the first report, the results obtained with Azotobacter chroococcum are presented in this paper.

The term "aerial-earth circuit" is employed here to designate the circuit between the atmosphere and the earth.

Hitherto the most biological investigations have been undertaken independent of the aerial-earth circuit or in other words, under the insulated condition. It is especially true with those concerned the microorganisms. Under the usual laboratory conditions, the microorganisms are insulated from the aerial-earth circuit so that the results obtained through such the procedure may not be the same as those in the closed circuit. Again it seems to be more evident in cases of the soil microorganisms whose habitat is the soil where the influence of such circuit as well as the earth potential is presumably marked.

Part I. Influence on Azotobacter chroococcum.

In view of these considerations, it was attempted to ascertain experimentally the influence of the aerial-earth circuit first on Azotobacter chroococcum which is well known organism for its interesting physiological activity, namely the fixation of atmospheric nitrogen.

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1) Reported in: The Proceedings of the Imperial Academy (Japan), IX, No. 2, 47–50, 1933; The Bulletin of the Agricultural Chemical Society of Japan, Vol. 9, Nos. 1—3, 31—37, January—March, 1933.

2) „ „: ibid. No. 7, 309—312, 1933; ibid. Nos. 7—9, 132—136, September, 1933.

Experimental Procedure.

An acclamatized, young culture of Azotobacter chroococcum in ASHBY's liquid medium was used to inoculate six flasks out of eight Erlenmeyer flasks (250 cc. volume) which contains 100 cc. of ASHBY's medium of the following properties, shown in Table I and II, in each, and the flasks were treated as described below:

Table I.

Chemical Properties of Ashby's Liquid Medium.

Composition.	Quantity.
Mannitol [$C_6H_8(OH)_6$].	10.0 g.
Magnesium sulfate ($MgSO_4 \cdot 7H_2O$).	0.2
Mono-potassium phosphate (KH_2PO_4).	0.2
Sodium chloride (NaCl).	0.2
Calcium sulfate ($CaSO_4 \cdot 2H_2O$).	0.1
Calcium carbonate ($CaCO_3$).	5.0
Distilled water.	1,000.0 cc.

Table II.

Physical Properties of Ashby's Liquid Medium.

Properties.	Values.
P_H	7.40
Specific gravity.	1.0077
Viscosity.	0.98984
Specific conductance.	0.00074
Surface tension.	79.559 (dynes per sq. cm.)
Osmotic pressure.	2.069

No previous record in regard to the physical properties of the medium is found so that they were determined realizing their significance in this investigation.

The flasks in the series were treated as follows:

Flask I. Two platinum electrodes (1.5×2.0 cm.) were inserted in the medium, and one of them was connected to an aerial antenna of twelve meters long and the other was earthed by a main water pipe. After the medium was inoculated with 1 cc. of 48 hours old culture, the flask was kept in an incubator at 30°C.

Flask II. Same as Flask I except it was not inoculated with the organism, and served as a control for Flask I in regard to the nitrogen fixation.

Flask III. Same as Flask I except no earth connection was made.

Flask IV. Same as Flask I but no connection to the antenna was made.

Flask V. Same as Flask IV but only one electrode was inserted and earthed.

Flask VI. Same as Flask IV except no earth connection was made.

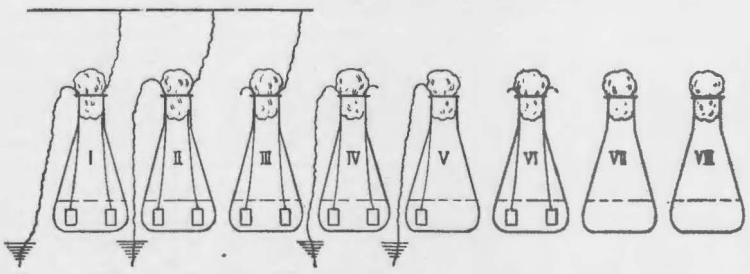
Flask VII. Same as Flask VI without insertion of any electrode.

Flask VIII. Same as Flask VII without inoculation, serving as the nitrogen control under the normal condition.

These arrangements are illustrated diagrammatically as follows:

Fig. I.

Diagrammatical Arrangement.



+Azot. −Azot. +Azot. +Azot. +Azot. +Azot. +Azot. −Azot.
(N-Control) (Normal Control) (N-Control)

The arrangement of the apparatus is also shown photographically in Plate I.

At the end of every 24 hours, with a few exceptions, the following observations were made:—

1. Macroscopical observations;
   a) The turbidity of culture.
   b) The formation of surface membrane.
2. Microscopical observations;
   a) The morphology of individual cell especially in regard to the cell division.
   b) The count of total cells was made by the use of hemocytometer and with a specially made cover glass in combination with Breed's ocular disc.

3. Nitrogen determination ;

At every five days intervals, the nitrogen was determined by PREGEL's micro-KJELDAHL method in the course of growth, and the final determination was made by the macro-KJELDAHL method for the total nitrogen.

Results.

1. Macroscopical observations :—

The macroscopical observations were made up to the tenth day as to the turbidity and the surface membrane, and the results together with some microscopical observations are given in Table III.

Table III.

Macroscopical and Microscopical Observations.

Number of Hours.	Number of Flasks.							
	I	II	III	IV	V	VI	VII	VIII
24	T ++ M ++ Y ++ P −	T − M − Y − P −	T + M ± Y + P −	T + M + Y + P −	T + M + Y + P −	T + M ± Y + P −	T + M ± Y + P −	T − M − Y − P −
48	T ++ M ++ Y ++ P −	T, M, Y, P ibid.	T ++ M + Y + P −	T ++ M ++ Y ++ P −	T ++ M ++ Y ++ P −	T ++ M + Y + P −	T ++ M + Y + P −	T, M, Y, P ibid.
72	T +++ M +++ Y +++ P −	T, M, Y, P ibid.	T ++ M ++ Y ++ P −	T ++ M ++ Y ++ P −	T ++ M ++ Y ++ P −	T ++ M ++ Y ++ P −	T ++ M ++ Y ++ P −	T, M, Y, P ibid.
96	T +++ M +++ Y +++ P −	T, M, Y, P ibid.	T +++ M ++ Y ++ P −	T +++ M +++ Y +++ P −	T +++ M +++ Y +++ P −	T +++ M ++ Y ++ P −	T +++ M ++ Y ++ P −	T, M, Y, P ibid.
120	T +++ M +++ Y +++ P −	T, M, Y, P ibid.	T +++ M ++ Y ++ P +	T +++ M +++ Y +++ P +	T +++ M +++ Y +++ P +.	T +++ M ++ Y ++ P +	T +++ M ++ Y ++ P +	T, M, Y, P ibid.
(Missed one day) 168	T, M, Y ibid. P ±	T, M, Y, P ibid.	T, M, Y ibid. P ++	T, M, Y ibid. P ++	T, M, Y ibid. P ++	T, M, Y ibid. P ++	T, M, Y ibid. P ++	T, M, Y, P ibid.
192	T, M, Y ibid. P +	T, M, Y, P ibid.	T, M, Y ibid. P +++	T, M, Y ibid. P ++	T, M, Y ibid. P ++	T, M, Y ibid. P ++	T, M, Y ibid. P ++	T, M, Y, P ibid.

Number of Hours.	Number of Flasks.							
	I	II	III	IV	V	VI	VII	VIII
216	T ⫼ M ⫼ Y ⫼ P ++	T — M — Y — P —	T ⫼ M ++ Y ++ P ⫼	T ⫼ M ⫼ Y ⫼ P ++	T ⫼ M ⫼ Y ⫼ P ++	T ⫼ M ++ Y ++ P ++	T ⫼ M ++ Y ++ P ++	T — M — Y — P —
240	T, M, Y ibid. P ++	T, M, Y, P ibid.	T, M, Y ibid. P ⫼	T, M, Y ibid. P ++	T, M, Y ibid. P ++	T, M, Y ibid. P ++	T, M, Y ibid. P ++	T, M, Y, P ibid.

Notes:— T, turbidity: M, membrane: Y, young, growing cells: P, pleomorphic cells: (The cells were observed through the microscope while the count was made.) ±, doubtful: —, no change or none: +, slight or few: ++, marked or many: ⫼, heavy or numerous: ⫼, very heavy or numerous:

Table III indicates that Flask I became turbid sooner than the rest, and those flasks which were earthed became more turbid than Flask VII which is the normal control. The formation of the surface membrane was in the same order as the turbidity. These results indicate plainly that the completion of circuit has a marked influence and also the earthing alone has a very beneficial influence on the growth of Azotobacter chroococcum. The stimulation effect was observed after 24 hours and at the end of 48 hours, the difference was noted very plainly.

2. *Microscopical observations :—*

The total number of cells was counted directly by means of microscope so that the morphological description of an individual cell was made at the same time. The results are given in Table IV while the conditions of cells were already noted in table III.

Table IV.

Growth of Azotobacter chroococcum at Different Age.

Number of Hours.	Number of Flasks.							
	I	II	III	IV	V	VI	VII	VIII
Initial.	366*	—	366*	366*	366*	366*	366*	—
24	12,112	—	5,746	6,288	5,887	5,112	5,232	—
48	35,211	—	12,154	21,408	23,183	13,883	12,957	—
72	38,028	—	18,352	24,873	28,535	18,197	17,549	—
96	44,028	—	20,985	28,676	29,661	20,197	19,605	—
120	45,915	—	26,662	30,140	30,028	27,323	26,000	—

Notes:— * Number of organisms per cc. in thousand.

Table IV indicates that the growth of Azotobacter chroococcum was influenced markedly by closing the circuit or earthing alone. The count after five days remained practically constant and the pleomorphic cells began to appear in some of the flasks making the correct counting difficult so that no further record is given after that. Flask I exceeded all the rest and Flask IV and V were much better than Flask III, VI and VII which were very similar. The stimulation began to show its influence after 24 hours. Also as it was noted in Table III, the young, multiplying cells were most numerous in Flask I throughout the investigation and the pleomorphic cells began to appear later than the rest, which seems to indicate that the closed circuit stimulates the cell metabolism. Again Flasks IV and V were better than the others in this respect, indicating that the earthing alone had some effect.

3. Physiological observation :—

Although the nitrogen determinations were made at every five days intervals by PREGEL'S micro-KJELDAHL method, here only the results obtained at the end of fifteen days by the ordinary KJELDAHL method for the total nitrogen will be given in Table V.

Table V.

Quantity of Nitrogen fixed.

Number of flasks.	Total nitrogen fixed in fifteen days per 100 cc. of medium.
I	4.099 mg.
II	——
III	2.830
IV	3.339
V	3.309
VI	2.680
VII	2.724
VIII	——

Table V indicates that much greater amount of nitrogen viz. more than one and one half times of the normal control, was fixed in Flask I where the aerial-earth circuit was closed, and even the earthing alone fixed much more than the normal control. The connection only to the antenna did not show any difference, as shown by the result in Flask III.

Discussions.

The nature of the stimulation which was observed in this investigation may be electrical in nature, but no previous investigation was undertaken in regard to

this phase of problem so far as the author is aware. The previous investigations which are concerned with the influence of electricity upon the microorganism as well as other organisms, seem to confine themselves, at least experimentally to electricity applied directly to the organisms, either galvanic or static form, and the influence observed by them may be considered as electro-chemical or the secondary influence in most cases.

The amount of electric current which passes through such a system as used in this investigation is governed primarily by the conductivity of the culture medium as well as the dielectrics of the system, and it must be a very small amount judging from the physical properties of the medium. No attempt however was made at this time to measure the current since it is very difficult to determine it accurately owing to the continuous change in the culture medium as the growth of the organism progresses as well as the irregularity of the atmospheric charge and the earth potential.

Since the physical properties of culture medium seem to have the great influence upon the physiological activities of organisms, they should receive more attention in the physiological investigation than ever before as well as the physical ecological factors.

Summary of Part I.

The influence of aerial-earth circuit on Azotobacter chroococcum was investigated by cultivating it in the closed aerial-earth circuit, connecting it either to the antenna or to the earth, and comparing the results against the normal control which was grown under the ordinary laboratory condition. From the results obtained, the following summary may be made:

(1) In the closed aerial-earth circuit, Azotobacter chroococcum was influenced markedly both culturally and physiologically. Its growth was more vigorous and fixed much more nitrogen than the rest.

(2) Connection to the antenna alone did not have any influence.

(3) Connection to the earth alone exerted better influence but was not so great as in the case of the closed circuit.

The principle underlying this investigation seems to be far reaching, scientifically as well as practically, and further investigations with various microorganisms are in progress.

Part II. Mechanism of the Influence on Azotobacter chroococcum as to its Potential.

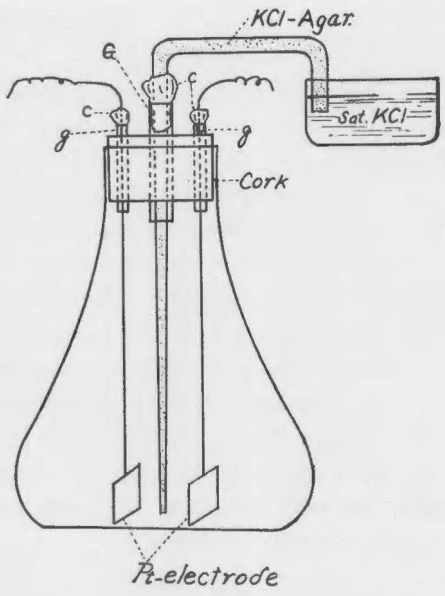
As Part I in this series of investigation, it was experimentally proven that the growth as well as the fixation of atmospheric nitrogen of Azotobacter chroococcum was activated in the closed aerial-earth circuit and even by earthing only. Subsequently an enquiry was made as to the mechanism of activation in regard to the potential of Azotobacter chroococcum culture, and the results are reported here as Part II.

Experimental Procedure.

The same strain of Azotobacter chroococcum and the medium were used as noted in the first report, but the flasks were equipped slightly differently so that the potential of the culture is determined easily and all the manipulations can be carried out aseptically, as shown in Fig. II.

Each flask was provided with a cork which has two glass tubes, g (4 mm. diameter) through either one or both, the platinum electrode is placed and held in position, and plugged with cotton. An another glass tube, G (8 mm. diameter) with cotton plug was provided through which a sample for P_H determination is taken and also an agar bridge is inserted when the potential is determined. The agar bridge is drawn to capillary at its end and considerably longer than the depth of the flask so that the tip can be broken off at every determination to keep it from contamination. The outer surface of the bridge is sterilized with alcohol and flamed before used. All through the investigation, very careful precautions were taken against contamination and agitation.

Fig. II.



The potential of Azotobacter chroococcum culture was determined against the steirle culture medium and also against the saturated calomel electrode by the following chains I. and II. respectively, using the K-type potentiometer, as shown in Plate II:

Pt (blank)	Sterile culture medium.	Sat. KCl (agar-bridge.)	Culture	Pt (blank).....(I)
Hg-HgCl	Sat. KCl	Sat. KCl (agar-bridge.)	Culture	Pt (blank).....(II)

The electromotive force thus determined, was converted into Eh, the standard E.M.F., or the normal hydrogen electrode potential. At the sametime, the hydrogen electrode potential was determined experimentally, and the difference between that and Eh is given so that the comparable intensity can be inferred.

Influence of Aerial-Earth Circuit on the Sterile Medium :

First it was attempted to ascertain if the potential of the sterile medium is influenced to any extent by the aerial-earth circuit, as follows ;

Four Erlenmeyer flasks with 100 cc. sterile ASHBY's solution were taken and treated in the following manner : Flask I. connected to the antenna and earthed ; Flask II. earthed only ; Flask III. connected to the antenna only ; Flask IV. no outside connection. The potential was determined at 24 hours intervals under aseptic conditions. The results are given in Table VI.

Table VI.

Influence of Aerial-Earth Circuit on the Sterile Medium.

Flasks.* \ Time in hours.		0	24	48	72	96	120
		(volts.)	(volts.)	(volts.)	(volts.)	(volts.)	(volts.)
I.	Potential determined.	0.1205	0.1454	0.1446	0.1558	0.1574	0.1566
	H_2-electrode potential.	0.9355	0.9360	0.9390	0.9440	0.9430	0.9410
	Eh.**	0.3675	0.3924	0.3916	0.4028	0.4044	0.4036
	Difference...	0.5680	0.5436	0.5474	0.5412	0.5386	0.5374
II.	Potential determined.	0.1257	0.1369	0.1383	0.1261	0.1439	0.1250
	H_2-electrode potential.	0.9315	0.9400	0.9440	0.9385	0.9400	0.9430
	Eh.	0.3737	0.3839	0.3853	0.3731	0.3909	0.3720
	Difference.....	0.5578	0.5561	0.5587	0.5654	0.5491	0.5710
III.	Potential determined.	0.1205	0.1478	0.1556	0.1653	0.1681	0.1589
	H_2-electrode potential.	0.9315	0.9410	0.9400	0.9440	0.9385	0.9440
	Eh.	0.3675	0.3948	0.4026	0.4123	0.4151	0.4059
	Difference.....	0.5640	0.5432	0.5374	0.5317	0.5234	0.5381
IV.	Potential determined.	0.1262	0.1296	0.1262	0.1280	0.1290	0.1285
	H_2-electrode potential.	0.9300	0.9410	0.9400	0.9465	0.9385	0.9207
	Eh.	0.3732	0.3766	0.3732	0.3750	0.3760	0.3757
	Difference.....	0.5568	0.5644	0.5668	0.5715	0.5625	0.5448

Notes :— * Flask I. antenna and earthed ; II. earthed only ; III. antenna only ; IV. no outside connection.

** Eh, the potential of medium converted into the standard, or the normal hydrogen electrode potential.

Table I indicates that the potential of Flask IV which is the control, was not changed to any extent, although in the other flasks, the potential increased slightly.

Influence of Aerial-Earth Circuit on the Potential of Azotobacter chroococcum Culture :

The potential of Azotobacter chroococcum culture against the sterile medium and also against the saturated calomel electrode, was determined as follows:

Four flasks A, B, C and D which were treated same as Flask I, II, III and IV respectively in the previous case, except all these flasks were inoculated with 1 cc. of 48 hours old culture of Azotobacter chroococcum. To determine the potential of the culture against the sterile medium, Flask D was chained against Flask IV. The results are shown in Table VII and Fig. III.

Table VII.

Influence of Aerial-Earth Circuit on the Potential of Azotobacter chroococcum Culture.

Flasks.* \ Time in hours.		0	24	48	72	96	120
		(volts.)	(volts.)	(volts.)	(volts.)	(volts.)	(volts.)
A.	Potential determined.	0.1612	0.0635	0.0906	0.0447	0.0262	0.0453
	H_2-electrode potential.	0.9315	0.9220	0.9340	0.9210	0.9205	0.9205
	Eh.**	0.4083	0.3155	0.3376	0.2917	0.2732	0.2923
	Difference.	0.5233	0.6065	0.5964	0.6293	0.6473	0.6282
B.	Potential determined.	0.1717	0.0650	0.0649	0.0331	0.0223	0.0308
	H_2-electrode potential.	0.9300	0.9140	0.9260	0.9320	0.9320	0.9320
	Eh.	0.4187	0.3120	0.3119	0.2801	0.2693	0.2778
	Difference.	0.5113	0.6020	0.6141	0.6519	0.6627	0.6542
C.	Potential determined.	0.1629	0.0514	0.0411	0.0083	−0.0234	−0.0307
	H_2-electrode potential.	0.9335	0.9190	0.9170	0.9140	0.9280	0.9280
	Eh.	0.4099	0.2984	0.2881	0.2553	0.2236	0.2163
	Difference.	0.5236	0.6206	0.6289	0.6587	0.7044	0.7117
D.	Potential determined.	0.1557	0.0496	0.0348	0.0009	−0.0127	−0.0193
	H_2-electrode potential.	0.9330	0.9180	0.9150	0.9150	0.9290	0.9280
	Eh.	0.4027	0.2966	0.2818	0.2479	0.2343	0.2277
	Difference.	0.5303	0.6214	0.6332	0.6671	0.6947	0.7003
D:IV.	Potential determined.**	0.0100	−0.0610	−0.0882	−0.0937	−0.1160	−0.1588
	Eh.	0.2570	0.1860	0.1588	0.1433	0.1310	0.0882

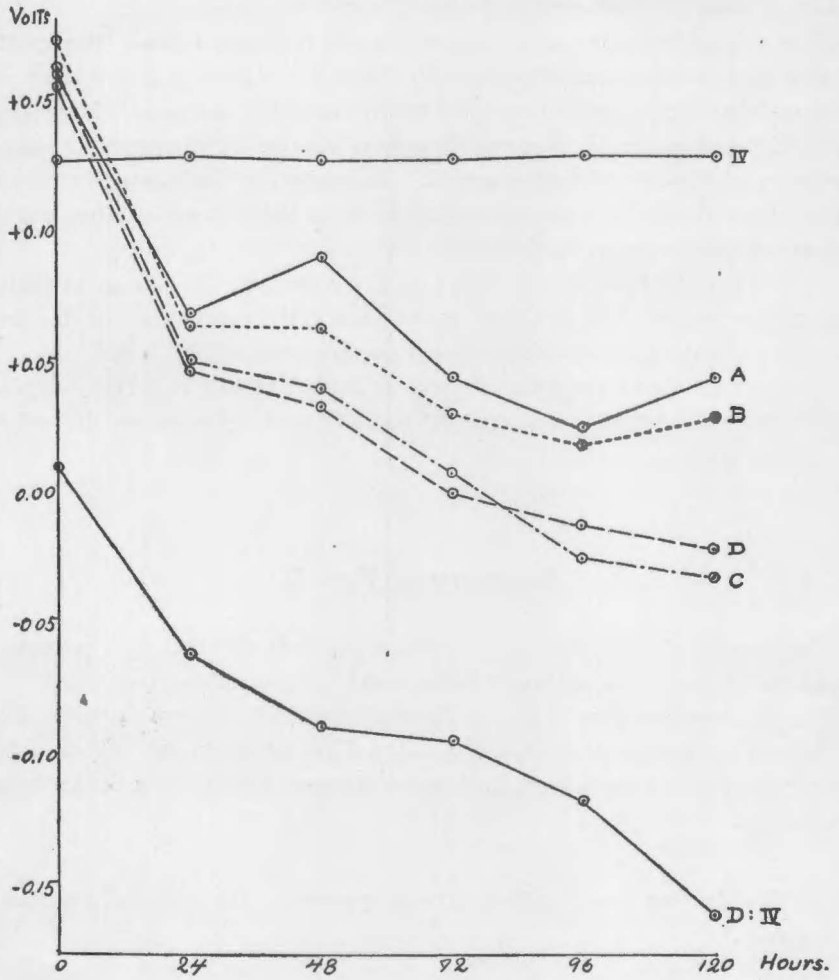
Notes:— * Flasks A, B, C and D were treated same as Flasks I, II, III and IV in Table VI respectively but inoculated.

** The potential of Flask D determined against Flask IV.

Table VII and Fig. III indicate that the potential difference is plainly determined between the culture and the sterile medium. The potential in all the inoculated flasks showed a sudden drop within the first 24 hours, but after that

Fig. III.

Potential of Azotobacter chroococcum under various Treatment.



Notes:— A.–antenna and earthed;
B.–earthed only;
C.–antenna only;
D.–normal control;
IV.–sterile medium.

the drop was different by the flask under different treatment. In Flask I, the least decrease of potential took place which was followed by Flask II while in Flask III and IV, the negative potential was observed.

Discussions.

So far as the author is aware, no previous investigation of this nature on Azotobacter chroococcum has ever been undertaken. Consequently it is very difficult to give a satisfactory interpretation of the results obtained.

As to the nature of potential measured in the bacterial culture, POTTER[1] who first measured the potential of Bact. coli in different media and also that of yeast, considered that such potential was electrical. However GILLESPIE[2] later demonstrated that such potential is a special case of the oxidation-reduction potential indicating the intensity of such reaction. Whatever may be the exact nature of such potential, it is certain that the potential is electrically measurable and such the measurement indicates the intensity of ionic activity in the culture.

In this investigation, it was attempted to ascertain, if such an intensity is influenced by the aerial-earth circuit or not, so that the mechanism of the activation obtained in the previous investigation may be understood.

So far as the data obtained and given in Table VII and Fig. III, it is plainly indicated that the different arrangement has different influence on the potential of Azotobacter culture.

Summary of Part II.

The results obtained in this investigation indicate that the potential of Azotobacter chroococcum culture is influenced by the aerial-earth circuit.

Considering these results in the light of those which were noted in Part I, the difference of potential which is due to the different treatment, may be a factor bringing about the difference in fixation of nitrogen and growth of Azotobacter chroococcum.

The further investigations are in progress and will be reported in future.

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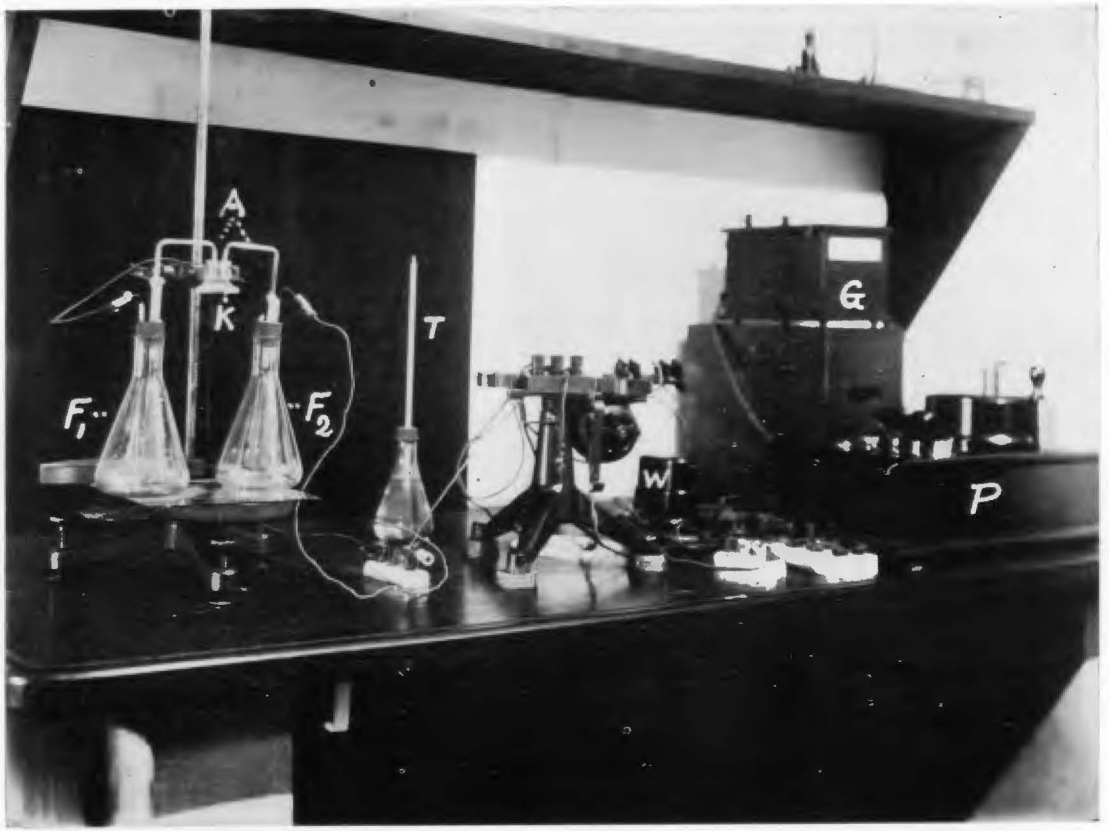
1) POTTER, M. C., Proc. Roy. Soc., London, Ser. B, 84, 260—276, 1911.

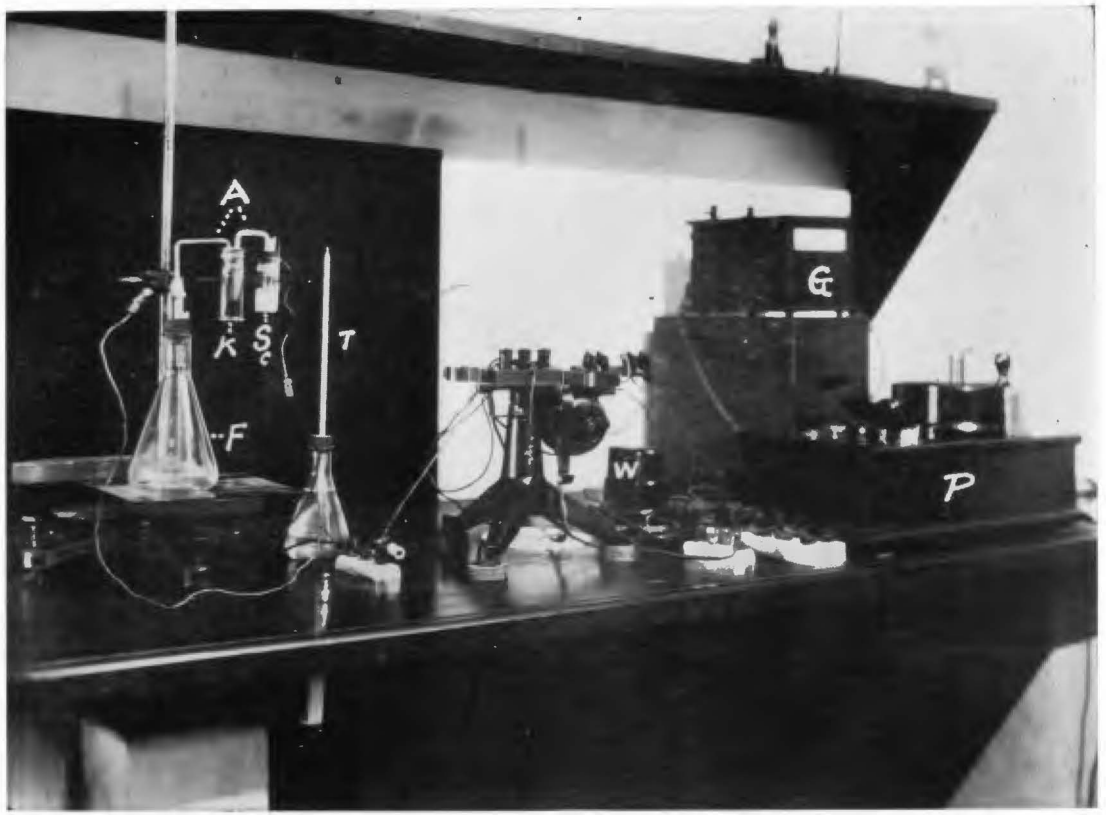
2) GILLESPIE, L. J., Soil Science, 9: 199—216, 1920.

PLATE I.



F1...flask with culture.
K...a small dish with saturated KCl.
T ..thermometer.
P...K-type potentiometer.
F2...flask with sterile culture medium.
A ..agar bridge.
G...galvanometer.
W...Weston standard cell.

PLATE II.



F...flask either with culture or sterile culture medium.
Sc...saturated calomel electrode.
Other parts are the same as in Plate I.