THE INFLUENCE OF NITRATE NITROGEN UPON THE GROWTH OF AND NITROGEN FIXATION BY AZOTOBACTER

by

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INTRODUCTION

Considerable information has been presented concerning the inhibiting effect of nitrate nitrogen upon nitrogen fixation by Azotobacter. According to Burk and Lineweaver (1930) the following investigators: "Bonazzi (1924), Zoond (1926), Kostyschew, Ryskaltschuk, and Schwezowa (1926) and others have shown that Azotobacter is able to utilize nitrates, ammonium salts, amino acids, and peptones, etc., in preference to free nitrogen, and that fixation is, indeed, not an essential function". In the same paper Burk and Lineweaver present proof, based upon data collected by suitable manometric methods, that nitrogen fixation is completely inhibited by the presence of adequate amounts of available nitrogen. They state, in conclusion, "the equilibrium concentration of rapidly available fixed nitrogen in the culture medium required to inhibit nitrogen fixation by Azotobacter completely is 0.5 mgm. per 100 c.c.". They have, therefore, supported and enlarged upon the views of previous workers "that fixation is a function resorted to only in the absence of sufficiently available fixed nitrogen". Burk, Lineweaver, and Horner (1934) stated that "a concentration of 0.0072 normal (100 p.p.m.), nitrate, am-

monia, or urea--when added to the culture medium--prevented all fixation of nitrogen, even when nitrogen gas was present as in air".

Regardless of the enormous volume of work concerning the influence of fixed nitrogen upon nitrogen fixation by Azotobacter, little attention has been given to the effect of continued growth of the organisms under conditions where fixation is completely inhibited. A survey of biological literature will reveal innumerable reports of instances wherein organisms adapted themselves to varied environments and thrived equally as well as, and in some cases, better than, in the environments in which they were found naturally. Moreover, in many such instances physiological processes necessary to the life of an organism in one environment was not essential in another, and as a part of the organism's adaptation to the second environment, the processes were discarded from its physiological make up. However, after the adaptation appeared to be complete, if the organism was again subjected to its original environment such discarded processes were regained provided the necessary stimulus was supplied, usually with greater rapidity than with which they were lost. Therefore, it is logical to reason that if a life process of Azotobacter were inhibited, for a sufficient time, it might be discarded; having lost

such a process, it is quite probable that some stimulating agent would be necessary to effect its resumption.

Previous studies (Briscoe, 1933) conducted in this laboratory showed that when certain cultures of Azotobacter were grown for a sufficient time, under conditions where nitrogen fixation was completely inhibited, then subjected to conditions where fixation was necessary, some strains failed to grow while others grew very poorly. This investigation was undertaken to determine, if possible, whether this abnormality resulted from a loss, on the part of the organisms, of the ability to metabolize free nitrogen; and, if so, whether this loss was temporary or permanent.

PLAN OF EXPERIMENT

This investigation was carried out in three parts: (1) a study of the effect of varying the concentration of nitrate nitrogen upon the growth of Azotobacter, (2) the effect of prolonged growth of Azotobacter at high nitrate concentrations upon the subsequent growth of the organisms, both in the presence and absence of fixed nitrogen, and (3) the influence of various concentrations of nitrate nitrogen upon nitrogen fixation by strains of Azotobacter that had been grown for a period of time under conditions of inhibited fixation.

METHODS

The cultures of Azotobacter employed in this experiment were isolated from soils collected near Manhattan, Kansas. All cultures studied grew readily upon the "N free" washed agar medium and produced a brown to black pigment, hence have been regarded as strains of the species Azotobacter chroococcum. Pure cultures were employed throughout this investigation, the purity of the cultures being carefully checked from time to time.

The cultural medium upon which the cultures were isolated, and which served as a basic solid medium, was a modification of Ashby's agar. Its composition was as follows:

$K_2HPO_4 \cdot 3H_2O$	2.5 gr.
$MgSO_4 \cdot 7H_2O$	0.2 gr.
NaCl	0.2 gr.
$CaCl_2$ (anhydrous)	0.05 gr.
$CaCO_3$	0.05 gr.
$FeCl_3$ (10% solution)	1 drop
Mannite	20.0 gr.
Agar agar (washed)	20.0 gr.
Distilled H_2O	1000 cc.

Washed agar was used in the preparation of all agar media--washed to free it as nearly as possible of all soluble material. The washing process consisted in suspending the agar-agar in distilled water, allowing it to settle for 24 hours, and removing the supernatant liquid. This process was repeated daily for a period of 10 days to 2 weeks. The agar media were prepared by diluting the equivalent of 20 grams of washed agar to 1000 c.c. with distilled water, and heating this mixture in the autoclave at 15 pounds pressure until the agar-agar was completely liquified. To the resulting colloidal agar-agar solution was added the other chemicals and the media sterilized for use.

These media were identical with those used in the liquid cultural media studies, except that in the latter the agar-agar was omitted. A favorable reaction was maintained in all instances by the addition of a slight excess of calcium carbonate. Such media, to which no fixed nitrogen was added, have been designated "N free" even though the liquid form contained in the vicinity of 1 p.p.m., while the agar contained approximately 6 p.p.m. nitrogen.

All strains of Azotobacter were cultured on the "N free" agar medium. All strains were also cultured on the "N free" medium, to which had been added increasing quan-

tities of nitrogen as potassium nitrate, to determine the highest concentration upon which the various strains would grow initially and continue growing. The highest potassium nitrate nitrogen concentration at which growth was good might be designated as the "initial nitrogen tolerance" of the strain in question. The tolerance of many cultures under investigation could be appreciably increased by allowing the cultures to grow at high concentrations of nitrogen for a period of time, then "stepping them up" to a slightly higher concentration. If this process were repeated several times quite a high tolerance could be attained by many cultures.

In general, the concentration which the cultures would tolerate also depended upon the frequency of transfer. If allowed to remain without transfer to fresh media for longer periods than 4 days, some of the cultures could not tolerate as high concentrations as if transferred every 3 or 4 days. When transferred every 3 or 4 days most cultures tolerated as high as 4000 p.p.m. nitrate nitrogen. While, if allowed to remain without transfer for as long as two weeks, relatively few of the cultures survived at concentrations above 1000 p.p.m. Therefore, during this investigation, unless otherwise stated, all cultures were transferred to fresh media every 3 or 4 days.

To determine the effect of growing Azotobacter at these higher concentrations of nitrate nitrogen, studies were made on 52 pure cultures. Each of these cultures was grown concurrently on the "N free" agar medium and on an agar medium containing the highest concentration of nitrate nitrogen at which it would grow "normally" when transferred every 3 or 4 days. After continued culturing for a chosen time the cultures were transferred from the high potassium nitrate nitrogen medium to the "N free" medium, where fixation was necessary for normal growth. At the same time they were transferred to a medium to which had been added 100 p.p.m. nitrate nitrogen (about the quantity normally fixed under these conditions). The relative visible growth on the two different media was observed.

The chief difficulty encountered was that an agar medium absolutely free of nitrogen could not be prepared, and slight growth might be expected to occur even if a culture had completely lost its ability to fix nitrogen. However, the nitrogen content of the washed agar was low enough to give valuable comparative results.

Time permitted a more extensive study of only a few of the cultures which gave indications of having difficulty in metabolizing free nitrogen after having grown at the high nitrate concentrations. These studies were made quantita-

tive in an effort to determine whether or not the cultures having grown at the high concentrations would fix nitrogen when cultured on the so-called "N free" medium and media of extremely low nitrogen contents. In this regard it was necessary to make numerous nitrogen determinations. Nitrate nitrogen determined by reduction with Davarda's alloy and distillation into standard acid; organic nitrogen by the official method as outlined by Association of Official Agriculture Chemists (1920).

The first quantitative studies were carried out by inoculating agar media with a water suspension of the culture (2 days old), and determining the nitrogen content after growth and upon inoculated but not incubated controls, the difference in the nitrogen contents of the two being regarded as nitrogen fixed. The inoculum consisted of a suspension of the organisms in distilled water, roughly standardized by matching with a McFarland's nephelometer tube No. 2. The plates were prepared by placing 50 c.c. of the sterile medium, of the desired nitrogen content, in large sterile Petri dishes (5½ in. dia.). Each plate was inoculated by spreading 0.3 c.c. of the suspension uniformly over the surface and the plates were incubated at 28°C. for 3 or 4 days, after which time the total nitrogen per plate was determined. The controls were inoculated in like manner

but the nitrogen determination made immediately after inoculation. During this phase of the study all samples were run in duplicate.

The results obtained from the studies conducted with the agar media indicated that the small amounts of nitrogen in the "N free" agar might suffice for limited growth and, hence, function as a stimulant for fixation. There was less nitrogen fixed at the end of 3 or 4 days when smaller quantities of nitrogen were initially present. Since it was impossible to prepare an agar medium with a minimum nitrogen content of less than 6 or 7 p.p.m., the studies were continued using a liquid medium of which the minimum nitrogen content was about 1 p.p.m.

One hundred c.c. portions of the liquid cultural media of the desired nitrogen content were placed in 300 c.c. Erlenmeyer flasks and inoculated. Since Azotobacter are strict aerobes, appreciable growth would be expected to occur only at the surface of the media. And since the media were almost devoid of colloidal material that might function as a catalyst, Waksman (1932) growth and fixation might be expected to take place at a time consuming rate. This was found to be the case, but the difficulty was partially overcome by continuously bubbling air through the cultures as suggested by Hunter (1923). The speed of aeration proved to

be an important factor in determining the velocity and extent of growth. It was found that if air was bubbled slowly through the cultures, growth was very poor and reached a maximum at about 4 days. On the other hand, when the cultures were vigorously aerated growth was comparatively rapid and increased in density until the medium was virtually depleted of energy material. With slow aeration, after about two days, the organisms appeared to flocculate and settle out, leaving the supernatant liquid virtually clear.

The data in Table I show the striking differences in nitrogen fixation of Azotobacter during varying incubation periods when subjected to rapid and slow aeration. Since the medium used was virtually nitrogen free, nitrogen fixation may also be considered a measure of growth. The values recorded are averages of duplicate samples.

Air, washed to free it of ammonia and other impurities, was forced into the culture through a glass tube which reached to the bottom of the Erlenmeyer flask. The lower extremity of the tube was sealed, then perforated laterally to facilitate a more even distribution of air through the culture. The tube was supported in the flask by a cotton stopper. This allowed the free escape of air and at the same time prevented contamination from without. The washing of the air consisted in passing it through a weak solu-

Table I. Comparative nitrogen fixation with slow and rapid aeration.

Time in days	Mg. nitrogen fixed per 100 c.c. cultural medium			
	Trial I		Trial II	
	Aeration slow	Aeration rapid	Aeration slow	Aeration rapid
2	0.53	2.38	0.72	1.15
4	2.16	3.58	1.24	3.46
6	1.85	4.54	--	--
7	--	--	1.53	4.16
8	1.96	4.72	--	--
9	--	--	1.83	5.22
11	--	--	1.60	5.27

tion of sodium hydroxide, a weak solution of sulphuric acid, and three containers of distilled water. The chief purpose of the water was to saturate the air with moisture and reduce evaporation from the culture.

Time permitted the extensive study of only one culture by the liquid cultural method. One drop of inoculum, prepared as previously described, was added to each flask. Fixation of nitrogen by this culture, after having grown for a definite period of time at 4000 p.p.m. nitrate nitro-

gen, was observed in the basic liquid medium to which had been added 0, 2½, 5, 10, 12½, 15, 17½, 20, 25, 50, 75, 100, and 125 p.p.m. nitrate nitrogen respectively. All samples were run in replications of from 2 to 5; in most cases 5. The cultures were incubated at 28°C. for varying periods of time (from 3 to 10 days), at the end of which time nitrogen determinations were made on all samples.

NITRATE NITROGEN AND GROWTH

It has been noted by previous workers that Azotobacter will tolerate a relatively high concentration of nitrate nitrogen. Sackett (1911) reported no harmful effect upon growth on agar media with concentrations as high as 825 p.p.m. nitrogen as $NaNO_3$. Briscoe (1933) observed that some cultures would grow well on agar media at concentrations as high as 3000 p.p.m. nitrate nitrogen. However, she reported that many cultures were apparently injured by this and even lower concentrations. Her studies indicated that the degree of nitrate injury depended largely upon the character of individual strains. Similar observations were made in a study of 24 cultures in this investigation.

The 24 cultures recorded in Tables II and III were isolated in late January, 1936. Each strain was transferred February 1, 1936, to the varying concentrations of nitrate

Table II. Growth of 24 cultures as observed April 15, 1936, after growing for 74 days (or 21 transfers) on agar with varying nitrogen contents; cultures transferred every 3 or 4 days beginning March 1.

Culture No.	Concentrations of Nitrate-N in p.p.m.									
	0	100	500	1000	2000	3000	4000	4500	5000	5500
1	4+	4+	4+	4+	4+	4+	3+	3+	1+	1+
1a	4+	4+	4+	4+	4+	4+	3+	3+	2+	2+
2	4+	4+	4+	4+	4+	4+	1+	?	2+	0
2a	4+	4+	4+	4+	4+	4+	4+	3+	1+	3+
2b	4+	4+	4+	4+	4+	4+	4+	3+	3+	3+
3	4+	4+	4+	4+	4+	4+	4+	2+	2+	0
3a	4+	4+	4+	4+	4+	4+	4+	3+	2+	1+
3b	4+	4+	4+	3+	2+	0	0	0	0	0
4	4+	4+	4+	4+	4+	4+	4+	3+	3+	2+
4a	4+	4+	4+	4+	4+	4+	3+	3+	0	0
4b	4+	4+	4+	4+	4+	4+	2+	3+	3+	0
4c	4+	4+	4+	4+	4+	4+	4+	3+	3+	2+
5	4+	4+	4+	4+	4+	4+	4+	4+	3+	3+
5a	4+	4+	4+	4+	4+	4+	4+	3+	2+	1+
5b	4+	4+	4+	4+	4+	4+	4+	4+	3+	1+
6	4+	4+	4+	4+	4+	4+	4+	0	0	0
6a	4+	4+	4+	4+	4+	4+	4+	3+	0	0
7	4+	4+	4+	4+	3+	4+	3+	3+	1+	1+
7a	4+	4+	4+	4+	1+	0	0	0	0	0
8	4+	4+	4+	4+	4+	4+	4+	4+	3+	3+
8a	4+	4+	4+	4+	4+	4+	4+	4+	4+	3+
8b	4+	4+	4+	4+	4+	4+	4+	4+	4+	3+
8c	4+	4+	4+	4+	4+	4+	4+	4+	4+	3+
9	4+	4+	4+	4+	4+	4+	4+	4+	4+	3+
	4+	4+	4+	4+	4+	4+	4+	4+	3+	3+

Table III. Growth of 24 cultures as observed June 3, 1936, after 123 days, (or 35 transfers) on agar with varying nitrogen contents.

Culture No.	Concentrations of Nitrate-N in p.p.m.									
	0	100	500	1000	2000	3000	4000	4500	5000	5500
1	4+	4+	4+	4+	4+	4+	4+	4+	4+	0
1a	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+
2	4+	4+	4+	4+	4+	4+	4+	0	0	0
2a	4+	4+	4+	4+	4+	4+	2+	4+	4+	0
2b	4+	4+	4+	4+	4+	4+	4+	4+	4+	0
3	4+	4+	4+	4+	4+	4+	1+	1+	1+	3+
3a	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+
3b	4+	4+	4+	4+	3+	0	0	0	0	0
4	4+	4+	4+	4+	4+	4+	4+	4+	4+	0
4a	4+	4+	4+	4+	4+	4+	4+	4+	4+	0
4b	4+	4+	4+	4+	4+	4+	4+	4+	3+	0
4c	4+	4+	4+	4+	4+	4+	4+	4+	3+	1+
5	4+	4+	4+	4+	4+	4+	4+	4+	4+	1+
5a	4+	4+	4+	4+	4+	4+	0	0	0	0
5b	4+	4+	4+	4+	4+	4+	4+	4+	4+	1+
6	4+	4+	4+	4+	4+	4+	4+	0	0	0
6a	4+	4+	4+	4+	4+	4+	4+	4+	0	0
7	4+	4+	4+	4+	4+	4+	4+	4+	4+	0
7a	4+	4+	4+	0	0	0	0	0	0	0
8	4+	4+	4+	4+	4+	4+	4+	4+	4+	0
8a	4+	4+	4+	4+	4+	4+	4+	4+	4+	0
8b	4+	4+	4+	4+	4+	4+	4+	4+	4+	0
8c	4+	4+	4+	4+	4+	4+	4+	4+	4+	0
9	4+	4+	4+	4+	4+	4+	4+	4+	4+	2+

nitrogen as KNO_3 indicated in the tables. The data in Table II indicate the relative growth of all cultures on each concentration after 21 transfers, or as observed on April 15. It may be noted that 17 of the 24 cultures grew normally at 4000 p.p.m. nitrate nitrogen. Only 2 strains failed to grow at this concentration, and neither of these grew normally above 1000 p.p.m.

Strains of Azotobacter vary greatly in the visible mass of growth produced upon a "N free" agar medium. Therefore, "normal" growth as applied to any strain in this discussion has reference to a visible mass of growth equal to that of the same strain maintained on the "N free" medium, and will be indicated in the tables as 4+. Relative growth will be indicated by 0, 1+, 2+, 3+, and 4+.

The data in Table III show the relative growth of the same cultures on agar with the same concentrations of nitrate nitrogen June 3, (49 days later) or after 35 transfers. It is evident from these data that no important variations in ability to grow have occurred during the 49 days. It should be noted that the cultures were transferred every 3 or 4 days. The frequency with which cultures are transferred is of major significance in determining their tolerance of high nitrate concentrations, as is indicated from the growth records of 24 cultures presented in Tables IV and V.

Table IV. Growth record of 2 day old Azotobacter cultures that remained untransferred during the previous 21 days.

Culture No.	Concentrations of Nitrate-N in p.p.m. agar medium						
	0	100	500	1000	2000	3000	4000
1	4+	4+	4+	4+	0	1+	0
1a	4+	4+	3+	0	3+	0	0
2	4+	4+	4+	4+	4+	4+	0
2a	4+	4+	4+	4+	4+	2+	2+
2b	4+	4+	4+	0	4+	0	0
3	4+	4+	4+	4+	0	0	0
3a	4+	4+	0	3+	0	3+	3+
3b	4+	4+	0	0	0	0	0
4	4+	4+	4+	3+	4+	4+	0
4a	4+	4+	4+	0	0	0	0
4b	4+	4+	4+	0	0	0	0
4c	4+	4+	4+	4+	4+	4+	3+
5	4+	4+	4+	4+	0	0	0
5a	4+	4+	4+	0	0	0	0
5b	4+	4+	4+	4+	4+	3+	1+
6	4+	4+	4+	4+	4+	4+	0
6a	4+	4+	4+	4+	0	0	0
7	4+	4+	0	0	0	0	0
7a	4+	4+	0	0	0	0	0
8	4+	4+	4+	4+	0	2+	1+
8a	4+	4+	4+	3+	0	0	0
8b	4+	4+	4+	4+	4+	0	0
8c	4+	4+	1+	0	0	0	0
9	4+	4+	4+	4+	4+	4+	4+

Table V. Growth records of 3 day old Azotobacter cultures that remained untransferred during the previous 33 days.

Culture No.	Concentrations of Nitrate-N in p.p.m. agar medium						
	0	100	500	1000	2000	3000	4000
1	4+	4+	1+	3+	0	0	0
1a	4+	4+	0	0	0	1+	1+
2	4+	4+	4+	0	4+	0	0
2a	4+	4+	4+	2+	2+	1+	1+
2b	4+	4+	0	0	0	0	1+
3	4+	4+	4+	1+	1+	1+	1+
3a	4+	4+	0	0	0	1+	1+
3b	4+	4+	0	0	0	0	0
4	4+	4+	4+	0	4+	1+	1+
4a	4+	4+	4+	0	0	0	0
4b	4+	4+	0	0	0	0	0
4c	4+	4+	4+	4+	4+	2+	2+
5	4+	4+	4+	0	0	0	0
5a	4+	4+	4+	0	0	0	0
5b	4+	4+	4+	0	0	0	0
6	4+	4+	4+	3+	2+	0	0
6a	4+	4+	4+	1+	0	0	0
7	4+	4+	0	0	0	0	0
7a	4+	4+	0	0	0	0	0
8	4+	4+	1+	0	0	0	1+
8a	4+	4+	0	0	0	0	0
8b	4+	4+	4+	0	0	0	0
8c	4+	4+	0	0	0	0	0
9	4+	4+	4+	3+	0	2+	0

The cultures upon which growth is recorded in Table IV were allowed to grow for 21 days without transfer, at which time they were transferred to freshly prepared media. Only 6 of the 24 cultures survived and only one grew normally at 4000 p.p.m.; and only 10 survived and 5 grew normally at 3000. Whereas, 20 survived and 18 grew normally at 500 p.p.m., and all grew normally at 100. In Table V is recorded the effect of a still longer interval without transfer (33 days). After 33 days without transfer still fewer cultures grew, particularly at the higher concentrations, upon being transferred to fresh media.

It is evident therefore, that if Azotobacter is to be grown on agar media at high concentrations of nitrate nitrogen, the transfer period is extremely important in maintaining growth. The initial tolerance is a measure of the subsequent tolerance only if the organisms are transferred often enough to eliminate this time-toxicity factor. The optimum transfer period was determined as being about 3 or 4 days.

THE INFLUENCE OF PROLONGED GROWTH IN PRESENCE OF NITRATE NITROGEN UPON THE ABILITY OF AZOTOBACTER TO GROW SUBSEQUENTLY IN THE PRESENCE AND ABSENCE OF FIXED NITROGEN

According to Burk, Lineweaver, and Horner (1934), if Azotobacter are grown in the presence of sufficient quantities of available nitrogen, all nitrogen fixation is inhibited, even in the presence of free nitrogen gas as in air. The equilibrium concentration of available nitrogen necessary to completely inhibit nitrogen fixation they found to be about 100 p.p.m. in a liquid cultural medium.

Although the equilibrium concentration for agar media was not determined in this investigation, the residual nitrate nitrogen was determined in agar media which initially contained 500 and 1000 p.p.m. nitrate nitrogen, and upon which Azotobacter had grown for 3 days. The quantitative data collected in this experiment are recorded in Table VI. It was assumed that the residual nitrate nitrogen recorded for culture 5b growing at 1000 p.p.m.(between 600 and 800 p.p.m.) was well in excess of the equilibrium concentration necessary to inhibit fixation, although the equilibrium concentration in agar media may be slightly different from that in a liquid. Therefore, since the experimental cul-

Table VI. Utilization of nitrates by culture 5b during 3 days incubation.

Calculated NO_3-N as KNO_3 added (p.p.m.)	Controls	After growing 6 months at 0 p.p.m. NO_3-N		After growing 6 months at 4000 p.p.m. NO_3-N	
	Mg. NO_3-N per 50 c.c. media determined	Mg. residual NO_3-N after 3 days growth	Mg. NO_3-N utilized	Mg. residual NO_3-N after 3 days growth	Mg. NO_3-N utilized
500	26.21	13.59	12.62	18.05	8.16
1000	52.35	39.68	12.67	34.23	18.12

tures used in this investigation tolerated nitrate nitrogen concentrations of 1000 p.p.m. and above on agar media, it was assumed that the growth of Azotobacter in the presence of such high nitrate concentrations was the equivalent of growth under conditions where fixation was completely inhibited.

Such a study was made upon 52 pure cultures. The concentrations of nitrate nitrogen as KNO_3 at which these cultures were grown were as recorded in Table VII. In all instances the concentration indicated represents a concentration where the culture grew well initially and continued "normal" growth when transferred every 3 or 4 days.

The cultures were grown under conditions of inhibited fixation for 105 days (30 transfers) to determine whether or not, by such treatment, a temporary or permanent change in the organism's ability to grow on "N free" media or to fix nitrogen could be effected.

After growing 3½ months at high nitrate concentrations all the cultures were transferred from the higher concentrations to agar media containing 0 and 100 p.p.m. nitrate nitrogen, respectively. At a concentration of 100 p.p.m. one might expect the organisms to grow equally as well as at the high concentrations, since this quantity of nitrate nitrogen is sufficient to furnish all the nitrogen needed for normal growth in the absence of fixation.

Table VII. Experimental cultures and the concentration of Nitrate-N at which they were grown.

Concentration of Nitrate-N at which cultured						
0	1000 p.p.m.		3000 p.p.m.		4000 p.p.m.	
All	K-1	C-4	E-2	E-1	D-3	O-2
	K-2	C-1	E-3	G-2	I-1	P-1
	H-1	C-2	G-4	G-3	I-2	P-3
	F-1	C-3	J-6	G-1	I-3	5b
	J-2	N-3	O-3	H-2	I-4	
	B-1	O-1	1	J-1	I-5	
	B-3	L-2	4	J-3	I-6	
	B-4		6a	L-1	J-5	
	H-3			N-5	N-2	
	A-4			N-2	N-1	
	F-2			M-1	N-4	

normal growth would occur at 0 p.p.m. only if the culture in question had maintained its nitrogen fixing powers.

In Table VIII are recorded in tabular form the cultures which grew markedly better at 100 than at 0 p.p.m. In all instances the growth was normal at 100 p.p.m. but only slight or visibly absent at 0 p.p.m. Since the "N free" agar medium contained from 6 to 7 p.p.m. nitrogen it is possible that this small quantity of nitrogen may have been adequate for the observed traces of growth at 0.

Table VIII. Cultures which failed to grow normally on "N free" agar but grew normally at 100 p.p.m. NO_3-N, after prolonged growth at high NO_3-N concentrations.

From 1000 p.p.m.	From 3000 p.p.m.	From 4000 p.p.m.
J-2	J-1	I-2
B-1		0-2
B-3	1	N-4
F-2	4	P-1
C-2	6a	P-3
L-2		5b*
5a		

*5b had grown at 4000 p.p.m. for about 7 months.

Of the 17 cultures showing marked differences, two typical examples were photographed after 48 hours growth. Figure 1 shows the growth of culture 5b on the two media, and Figure 2 that of culture I-2.

It would appear from this study that in the case of 17 of the 52 cultures some change in the physiology of the organisms has been effected. When such cultures were placed under conditions where nitrogen fixation was necessary for normal growth, they were apparently unable to utilize free atmospheric nitrogen, to any appreciable extent, in their metabolism. Quantitative measurements of the nitrogen fixed were conducted in an effort to reveal further the behavior of certain of these cultures.

INFLUENCE OF NITRATE NITROGEN UPON FIXATION

Time permitted of the detailed quantitative study of only a few of the 17 cultures which gave good qualitative evidence of having undergone alterations in ability to grow at 0 nitrogen, following culturing at high concentrations. Quantitative studies of nitrogen fixed on agar media in the absence of nitrates, and in the presence of varying quantities of nitrates, were made on culture 5b. In Tables IX, X, and XI are recorded the results of three experiments of this nature.

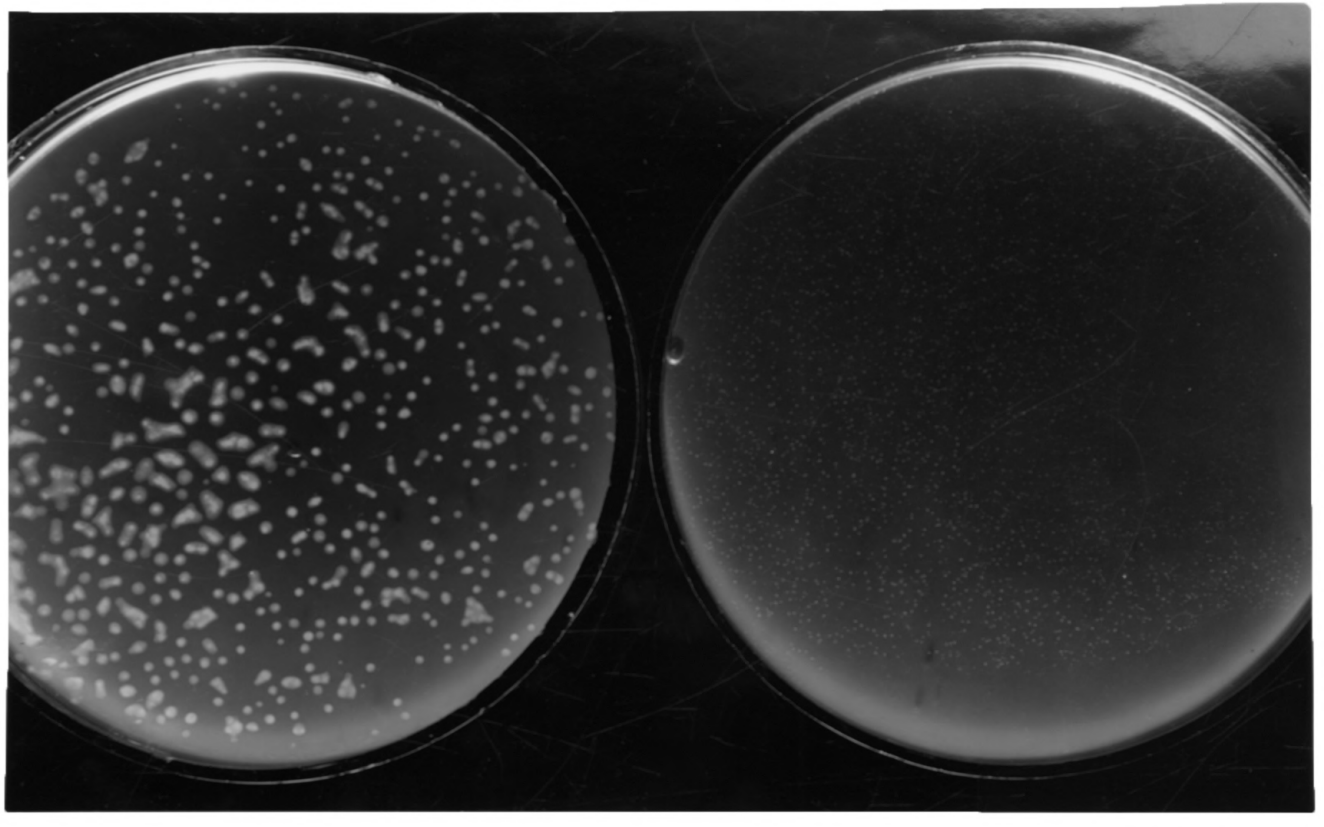

Figure 1. Left, growth of culture 5b (48 hrs.) at 100 p.p.m. nitrate nitrogen.
Right, growth of culture 5b (48 hrs.) at 0 p.p.m. nitrate nitrogen.

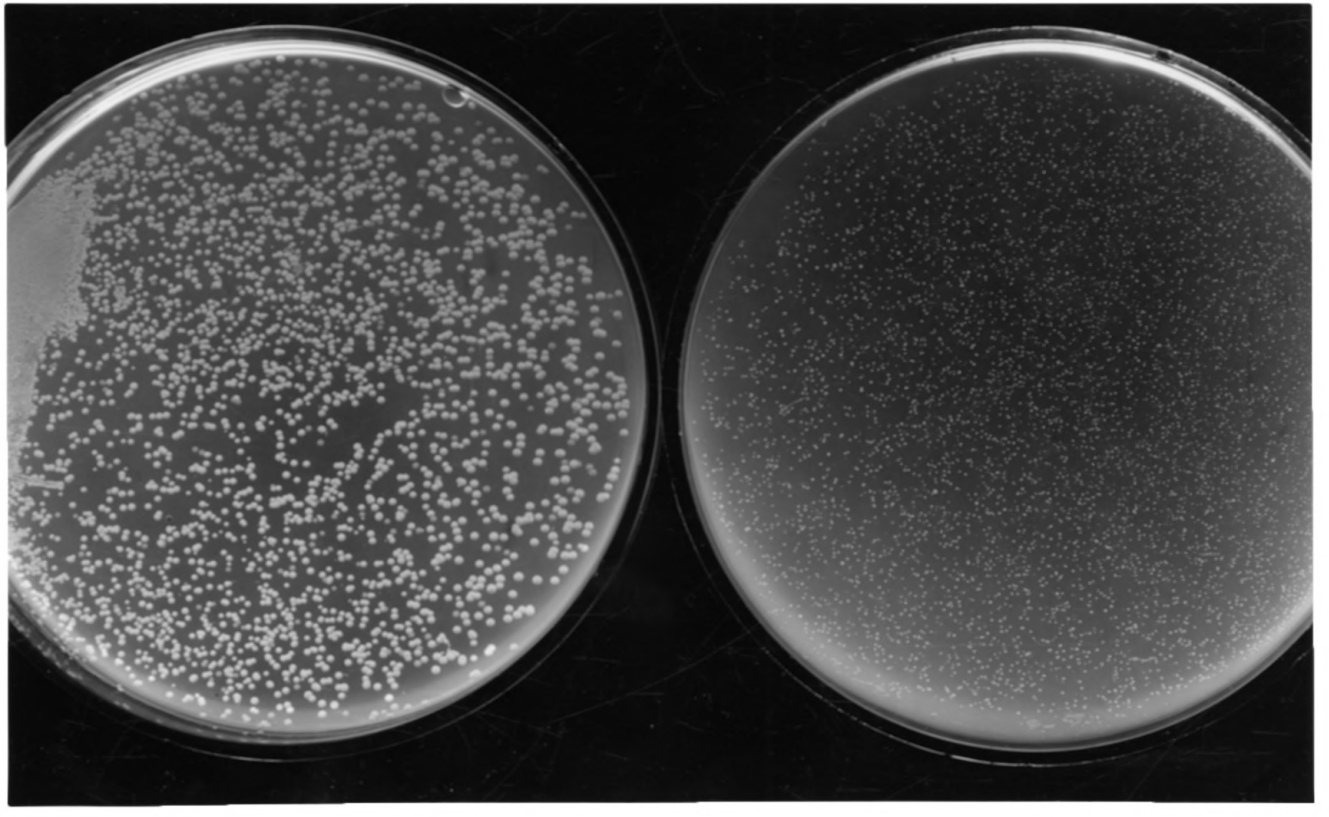

Figure 2. Left, growth of culture I-2 (48 hrs.) at 100 p.p.m. nitrate nitrogen.
Right, growth of culture I-2 (48 hrs.) at 0 p.p.m. nitrate nitrogen.

Table IX. Growth and nitrogen fixed by culture 5b, on agar of varying nitrogen contents; incubation period 3 days.

Calculated nitrate added to media (in p.p.m.)	Controls		"4000 culture" [1]				"0 culture" [2]			
	Mg. NO_3-N per 50 c.c. media determined	Mg. total N per 50 c.c. media determined	Observed growth	Total organic N per 50 c.c. media after growth	Mg. N fixed per 50 c.c. media	P	Observed growth	Total organic N per 50 c.c. media after growth	Mg. N fixed per 50 c.c. media	P
0	0.00	0.45	Tr	0.50	0.05	.4	4+	3.39	2.84	.01
50	2.39	2.84	2+	3.23	0.39	.8	4+	5.39	2.55	.01
100	4.80	5.25	3+	6.20	0.95	.01	4+	6.50	1.25	.01
150	7.22	7.67	4+	8.21	0.54	.3	4+	9.00	1.33	.01

1. "4000 culture" - the culture previously grown at 4000 p.p.m. NO_3-N

2. "0" culture" - the culture previously grown at 0 p.p.m. NO_3-N

Table X. Growth and nitrogen fixed by culture 5b, on agar of varying nitrogen contents; incubation period 3½ days.

Calculated NO_3-N added to media (in p.p.m.)	Controls		"4000 culture"				"0 culture"			
	Mg. NO_3-N per 50 c.c. media determined	Mg. total N per 50 c.c. media determined	Observed growth	Total organic N per 50 c.c. media after growth	Mg. N fixed per 50 c.c. media	P	Observed growth	Total organic N per 50 c.c. media after growth	Mg. N fixed per 50 c.c. media	P
0	0.00	0.48	1+	1.26	0.78	.01	4+	4.41	3.93	.01
50	2.49	2.97	3+	6.17	3.20	.01	4+	6.34	3.39	.01
100	5.01	5.74	4+	7.29	1.55	.01	4+	7.71	1.97	.01

Table XI. Growth and nitrogen fixed by culture 5b, on agar of varying nitrogen contents; incubation period 3 days.

Calculated NO_3-N added to media (in p.p.m.)	Controls		"4000 culture"				"0 culture"			
	Mg. NO_3-N per 50 c.c. media determined	Mg. total N per 50 c.c. media determined	Observed growth	Total organic N per 50 c.c. media after growth	Mg. N fixed per 50 c.c. media	P	Observed growth	Total organic N per 50 c.c. media after growth	Mg.N fixed per 50c.c. media	P
0	0	0.40	Tr	0.56	0.16	.2	4+	5.61	5.21	.01
10	0.51	0.91	1+	1.26	0.35	.05	--	--	--	
25	1.36	1.76	2+	2.51	1.15	.01	--	--	--	
50	2.62	3.02	3+	4.07	1.05	.01	--	--	--	
100	5.46	5.86	4+	6.75	0.89	.01	--	--	--	
150	7.74	8.14	4+	9.06	0.92	.01	--	--	--	

In these tables, and others to follow, are recorded in parallel the observed mean relative growth, total nitrogen metabolized, and the free atmospheric nitrogen metabolized by strains of the same culture grown at 0 and at 4000 p.p.m. nitrate nitrogen for relatively long periods of time. It is this last value, i.e., nitrogen fixed, that has been regarded as of special importance in this study; hence, the significance of the quantities of nitrogen fixed has been evaluated according to Fisher (1934). This significance is recorded in terms of P, where P represents the chance probability of the occurrence of a difference between two means, i.e., the mean total nitrogen present in the controls and the mean total nitrogen in the experimental cultures after growth, as great as that observed. P, therefore, is a measure of the significance of nitrogen fixed. For example, if for a recorded value P=0.4, then the probability is that 4 times out of 10 a value equal to that recorded will be due to chance. Values (differences) for which P is greater than 0.05 are not regarded by Fisher as being significant.

From Table IX it may be noted that culture 5b, after growing 7 months at 4000 p.p.m. nitrate nitrogen ("4000 culture"), failed to fix significant quantities of nitrogen at 0 and 50 p.p.m. in 3 days time, while fixation at 100

p.p.m. was quite definite. The same culture after growing at 0 p.p.m. for 7 months ("0 culture") fixed comparatively large quantities of nitrogen at all concentrations recorded. Table X shows the results secured from the same culture under the same conditions except that it was allowed to grow for 3½ days. All values of nitrogen fixed are clearly significant, but the difference between fixation by the "4000 culture" and the "0 culture" are quite striking; also the quantity fixed at 50 p.p.m. by the "4000 culture" is strikingly greater than that fixed at 0. In Table XI are recorded the results of a third trial, where at 3 days the "4000 culture" showed no significant fixation at 0 but the fixation at all nitrate concentrations tested is clearly significant. Again definite fixation by the "0 culture" at 0 nitrate may be noted.

Similar quantitative studies were made with culture I-2, the results of which are recorded in Tables XII, XIII, and XIV. Although this culture exhibited significant fixation in all cases, the comparative quantities of nitrogen fixed by the "0 culture" and the "4000 culture" are of great interest. In all 3 trials the fixation at 0 p.p.m. by the "0 culture" was strikingly greater than the fixation at the same concentration by the "4000 culture". Also, in all cases fixation by the "4000 culture" was strikingly greater

Table XII. Growth and nitrogen fixation by culture I-2, on agar of the following nitrogen contents, incubation period 3 days.

Calculated NO_3-N added to media (in p.p.m.)	Controls		"4000 culture"				"0 culture"			
	Mg. NO_3-N per 50 c.c. media determined	Mg. total N per 50 c.c. media determined	Observed growth	Total organic N per 50 c.c. media after growth	Mg. N fixed per 50 c.c. media	P	Observed growth	Total organic N per 50 c.c. media after growth	Mg. N fixed per 50 c.c. media	P
0	0.00	0.45	1+	1.29	0.84	.01	4+	6.35	5.90	.01
50	2.39	2.84	4+	4.31	1.47	.01	4+	5.94	3.10	.01
100	4.80	5.25	4+	6.66	1.41	.01	4+	7.08	1.83	.01
150	7.22	7.67	4+	8.03	0.36	.05	4+	9.00	1.33	.01

Table XIII. Growth and nitrogen fixation by culture I-2, on agar of the following nitrogen contents, incubation period 3 days.

Calculated NO_3-N added to media (in p.p.m.)	Controls		"4000 culture"				"0 culture"			
	Mg. NO_3-N per 50 c.c. media determined	Mg. total N per 50 c.c. media determined	Observed growth	Total organic N per 50 c.c. media after growth	Mg. N fixed per 50 c.c. media	P	Observed growth	Total organic N per 50 c.c. media after growth	Mg. N fixed per 50 c.c. media	P
0	0.00	0.48	1+	2.07	1.59	.01	4+	8.34	7.86	.01
50	2.49	2.97	3+	5.33	2.36	.01	4+	7.01	4.04	.01
100	5.01	5.73	4+	6.93	1.20	.01	4+	7.50	1.77	.01

Table XIV. Growth and nitrogen fixation by culture I-2, on agar of the following nitrogen contents, incubation period 3 days.

Calculated NO_3-N added to media (in p.p.m.)	Controls		"4000 culture"				"0 culture"			
	Mg. NO_3-N per 50 c.c. media determined	Mg. total N per 50 c.c. media determined	Observed growth	Total organic N per 50 c.c. media after growth	Mg. N fixed per 50 c.c. media	P	Observed growth	Total organic N per 50 c.c. media after growth	Mg. N fixed per 50 c.c. media	P
0	0.00	0.40	1+	0.84	0.44	.01	4+	10.02	9.62	.01
10	0.51	0.91	2+	1.47	0.56	.01	--	--	--	
25	1.36	1.76	3+	3.12	1.36	.01	--	--	--	
50	2.62	3.02	4+	5.55	2.53	.01	--	--	--	
100	5.46	5.86	4+	8.62	2.76	.01	--	--	--	
150	7.74	8.14	4+	10.31	2.17	.01	--	--	--	

at the lower concentrations (10 to 50 p.p.m.) of nitrate nitrogen than it was at 0.

From these studies two facts appear evident; first, in the absence of nitrate nitrogen the nitrogen fixing activity of the cultures grown at 0 concentration of nitrate nitrogen exceeds by far the nitrogen fixing activity of the cultures having been grown at the high concentrations. Second, small quantities of nitrate nitrogen appreciably stimulate fixation by the cultures previously grown at the high concentrations of fixed nitrogen.

It may be noted that the agar medium to which no nitrate was added contained appreciable quantities of nitrogen. This is nitrogen that remained in the agar after it had been subjected to the thorough washing as previously described. The form of this nitrogen was questionable; but since fixation by the cultures previously grown at the high concentrations was stimulated by relatively small quantities of nitrate nitrogen, it was thought this residual nitrogen might be active also in stimulating fixation. Consequently studies were continued using, instead of the agar media, liquid media in which the nitrogen concentration of the "N free" medium was considerably lower.

In Tables XV to XX inclusive are recorded the results of 6 separate experiments with culture 5b grown in a liquid

Table XV. Growth and nitrogen fixation by culture 5b in liquid media of varying nitrogen contents, incubation period 4 days.

Calculated NO_3-N added (in p.p.m.)	Controls		"4000 culture"			
	Mg. NO_3-N per 100 c.c. media determined	Mg. total N per 100 c.c. media determined	Observed growth	Total organic N per 100 c.c. media after growth	Mg. N fixed per 100 c.c. media	P
0	0	0.15	0	0.21	0.06	.3
0	0	0.15	0	0.18	0.03	.6
5	0.49	0.64	2+	0.89	0.25	.05
10	1.01	1.16	4+	2.03	0.87	.01
20	2.17	2.32	4+	3.50	1.18	.01
			"0 culture"			
0	0	0.15	4+	4.31	4.16	.01

Table XVI. Growth and nitrogen fixation by culture 5b in liquid media of varying nitrogen contents, incubation period 4 days.

Calculated NO_3-N added (in p.p.m.)	Controls		"4000 culture"			
	Mg. NO_3-N per 100 c.c. media determined	Mg. total N per 100 c.c. media determined	Observed growth	Total Organic N per 100 c.c. media after growth	Mg. N fixed per 100 c.c. media	P
0	0	0.15	0	0.20	0.05	.1
10	1.11	1.26	3+	3.03	1.77	.01
15	1.41	1.56	4+	3.67	2.11	.01
20	2.03	2.18	4+	3.70	1.52	.01
25	2.57	2.72	4+	4.21	1.49	.01
50	5.13	5.28	4+	5.94	0.66	.01
75	7.54	7.69	4+	7.92	0.23	.3
100	9.97	10.12	4+	10.34	0.22	.4
125	12.77	12.92	4+	12.77	-0.15	
			"0 culture"			
0	0	0.15	4+	3.06	2.91	.01

Table XVII. Growth and nitrogen fixation by culture 5b in liquid media of varying nitrogen contents, incubation period 4 days.

Calculated NO_3-N added (in p.p.m.)	Controls		"4000 culture"			
	Mg. NO_3-N per 100 c.c. media determined	Mg. total N per 100 c.c. media determined	Observed growth	Total Organic N per 100 c.c. media after growth	Mg. N fixed per 100 c.c. media	P
0	0.00	0.09	0	0.13	0.04	.05
2½	0.25	0.34	2+	0.52	0.18	.01
5	0.50	0.59	3+	1.40	0.81	.01
7½	0.75	0.84	3+	1.40	0.56	.01
10	1.00	1.09	3+	1.40	0.31	.02
12½	1.25	1.34	3+	1.52	0.18	.04
15	1.50	1.59	3+	1.83	0.24	.03
17½	1.75	1.84	3+	2.10	0.26	.01

Table XVIII. Growth and nitrogen fixation by culture 5b in liquid media of varying nitrogen contents, incubation period 5 days.

Calculated NO_3-N added (in p.p.m.)	Controls		"4000 culture"			
	Mg. NO_3-N per 100 c.c. media determined	Mg. total N per 100 c.c. media determined	Observed growth	Total organic N per 100 c.c. media after growth	Mg. N fixed per 100 c.c. media	P
0	0.00	0.09	0	0.15	0.06	.2
$2\frac{1}{2}$	0.25	0.34	4+	1.86	1.52	.01
5	0.50	0.59	4+	2.23	1.64	.01
$7\frac{1}{2}$	0.75	0.84	4+	2.63	1.79	.01
10	1.00	1.09	4+	2.93	1.84	.01
$12\frac{1}{2}$	1.25	1.34	4+	3.07	1.73	.01
15	1.50	1.59	4+	2.23	0.64	.01
			"0 culture"			
0	0.00	0.09	4+	5.38	5.29	.01

Table XIX. Growth and nitrogen fixation by culture 5b in liquid media of varying nitrogen contents, incubation period 5 days.

Calculated NO_3-N added (in p.p.m.)	Controls		"4000 culture"			
	Mg. NO_3-N per 100 c.c. media determined	Mg. total N per 100 c.c. media determined	Observed growth	Total organic N per 100 c.c. media after growth	Mg. N fixed per 100 c.c. media	P
0	0.00	0.09	Tr	0.21	0.12	.01
2½	0.25	0.34	3+	1.28	0.94	.01
5	0.50	0.59	4+	2.11	1.52	.01
7½	0.75	0.84	4+	2.40	1.56	.01
10	1.00	1.09	4+	1.72	0.63	.01
12½	1.25	1.34	4+	2.10	0.66	.01
15	1.50	1.59	4+	2.29	0.45	.01
			"0 culture"			
0	0.00	0.09	4+	4.85	4.76	.01

Table XX. Growth and nitrogen fixation by culture 5b in liquid media of varying nitrogen contents, incubation period 8 days.

Calculated NO_3-N added (in p.p.m.)	Controls		"4000 culture"			
	Mg. NO -N per 100 c.c. media determined	Mg. total N per 100 c.c. media determined	Observed growth	Total organic N per 100 c.c. media after growth	Mg. N fixed per 100 c.c. media	P
0	0.00	0.09	1+	1.09	1.00	.01
2½	0.25	0.34	4+	3.20	2.86	.01
5	0.50	0.59	4+	3.00	2.41	.01
7½	0.75	0.84	4+	4.06	3.22	.01
10	1.00	1.09	4+	3.44	2.35	.01
12½	1.25	1.34	4+	4.27	2.93	.01
15	1.50	1.59	4+	4.67	2.83	.01
				"0 culture"		
0	0.00	0.09	4+	5.80	5.71	.01

medium containing varying quantities of nitrate nitrogen. Table XV, representing the first trial, shows that the "4000 culture" did not fix significant quantities of nitrogen at 0 p.p.m. in 4 days, while at low nitrate concentrations it exhibited definite fixation. Moreover, fixation by the "0 culture" was clearly significant at 0. Similar results were obtained in trial 2 recorded in Table XVI. However, in this instance the "4000 culture" was cultured at concentrations increasing from 0 to 125 p.p.m. It may be observed that concentrations ranging from 10 to 25 p.p.m. showed little difference as to the stimulating effect of fixed nitrogen upon nitrogen fixation. In this instance at concentrations of about 50 p.p.m. the inhibitory effect of available nitrogen upon nitrogen fixation began to operate, and fixation decreased with increasing nitrogen content until at 125 p.p.m. no fixation at all occurred. This is in accordance with observations of previous workers.

A third experiment, the data of which are recorded in Table XVII, gave comparable results to trials 1 and 2, in that fixation by the "4000 culture" was not significant at 0 p.p.m., while the small addition of only $2\frac{1}{2}$ p.p.m. stimulated the culture to fix a significant quantity of nitrogen. In trial 4, see Table XVIII, the culture was allowed to grow for 5 days, however, the same general results were

obtained. A fifth trial, consult Table XIX, in which the culture was allowed to grow for 5 days, showed significant fixation at 0 by the "4000 culture". However, this fixation was in no way comparable to that recorded at 0 for the "0 culture".

The data obtained in trial 5 were interpreted as indicating that if the culture previously grown at 4000 p.p.m. nitrate-N were allowed to grow for a longer time at 0 it might fix appreciable quantities of nitrogen. This was found to be the case, as shown by a sixth trial (Table XX) in which the culture was allowed to grow for 8 days. Still fixation at 0 p.p.m. nitrogen by the "4000 culture" is less than one-fifth the fixation by the "0 culture" at the same concentration.

GENERAL DISCUSSION

The physiologist is aware that an organism, whether it be simple or complex in structure, if subjected to a slightly varied environment from that in which it normally exists, will in many cases adapt itself to the new environment and thrive equally as well as in the old. Also, if in the new environment some process, essential to normal activity in the old environment, is not needed the tendency is for this process to gradually become ineffective and perhaps finally

disappear altogether. Because of the very short generation time, being measured in terms of minutes, such alterations are known to occur among bacteria with remarkable rapidity.

Previous workers (Burk, Lineweaver, and Horner (1934) and others) have noted that nitrogen fixation is a function of Azotobacter resorted to only in the absence of sufficient quantities of readily available fixed nitrogen to supply it with the immediate needs for normal growth. It has also been noted by some previous investigators that in most cases Azotobacter will tolerate concentrations of nitrate nitrogen, and similar forms of available nitrogen, far in excess of the concentrations necessary to inhibit nitrogen fixation. However, some have observed that this tolerance varies with different strains and in many cases the variation is quite marked.

Sackett (1911) reported no harmful effect upon growth of Azotobacter of nitrogen concentrations as high as 825 p.p.m. as $NaNO_3$. Briscoe (1933) found that various strains of Azotobacter would grow normally at concentrations of nitrate nitrogen as high as 3000 p.p.m., while others refused to grow at much lower concentrations. She also reported a variation in the adaptability of certain strains to the high concentrations.

The findings in this investigation are perfectly in accord with those cited above. Considerable variation was exhibited among the cultures employed as to their tolerance of and adaptability to the higher concentrations of nitrate nitrogen. In most cases, however, it was found that the cultures employed would tolerate concentrations of 1000 p.p.m. or greater. In many cases concentrations as great as 4000 p.p.m. did not prove noxious to growth. It has already been pointed out that these concentrations of nitrate nitrogen probably completely eliminate fixation.

Hence, it was possible to grow Azotobacter under conditions where fixation of nitrogen was inhibited and yet where macroscopic growth was normal. Briscoe (1933) reported that some cultures when grown under these conditions for sufficient time after which they were again placed under conditions where fixation was necessary for normal growth, failed to grow--others grew poorly, while many grew normally. This observation has been supported and enlarged upon in this investigation.

Cultures of Azotobacter upon which the data are recorded were grown under conditions of inhibited fixation for an extended time after which they were subjected to conditions where growth depended upon nitrogen fixation. In many cases growth was not normal and in some cases no vis-

ible growth occurred.

Furthermore, extensive quantitative studies upon one culture previously grown under conditions of inhibited fixation, revealed that the culture actually failed to fix significant quantities of nitrogen in four days time in a medium virtually free of combined nitrogen. The same culture, however, after growing on a "N free" medium for the same extended period showed good growth and quantitative fixation at 4 days in an identical medium.

In connection with these quantitative studies it was established that small amounts of nitrates when added to the media materially stimulated nitrogen fixation by the culture previously grown under conditions of inhibited fixation. Furthermore, it was observed that if the culture which had been grown under conditions of inhibited fixation was grown in the "N free" medium longer than 4 days, say 8 days, significant fixation would occur. It is possible that this observed fixation after 4 days results from the effect of the traces of nitrogen in the medium. It has been established that small amounts of nitrate nitrogen stimulate fixation materially and it may be that the very small quantities of nitrogen in the "N free" medium are sufficient to function in this way. Fixation once started may be expected to operate as an accumulative process and eventually

nitrogen will be present in sufficient quantities for maximum stimulation. In other words, since nitrate nitrogen stimulates fixation by the "4000 culture" and since it is impossible to obtain a medium devoid of traces of available nitrogen, it would be illogical to expect that nitrogen fixation would not eventually occur in such a medium. Fortunately, however, the nitrogen content of the liquid medium employed was so low that significant fixation did not occur until after 4 days time.

According to Burk and Lineweaver (1934) "Azotobacter possesses a highly specific enzyme system, azotase, capable of catalyzing the fixation of nitrogen gas at ordinary temperatures and pressures". They state that "nitrogen gas is ordinarily fixed by Azotobacter in intracellular form only, the amount being directly proportional to the amount of growth".

Considering nitrogen fixation as an enzymatic process, it is possible to offer an explanation for the results obtained in this investigation. Azotobacter growing at high nitrogen concentrations would have no need for the nitrogen fixing enzyme. Its synthesis, under such conditions,might be expected to be reduced to a minimum or possibly discontinued entirely, yet the organism would probably retain

the ability to synthesize the enzyme much longer than it would actually continue to synthesize it. The cells making up a culture grown on a medium of high nitrate content would, under such conditions, become depleted of the enzyme and, hence, when transferred to a medium virtually free of nitrogen would be incapable of immediately fixing nitrogen. If, however, the ability to synthesize the enzyme were retained and the conditions were favorable, the necessary constituents being present, it would soon again become an active nitrogen fixing culture. It is probable that nitrogen is an essential constituent of the enzyme and when present in such small quantities as in the nitrogen free medium both growth and synthesis of the necessary enzyme would of necessity have to be extremely slow.

A second explanation for the stimulating effect of fixed nitrogen is that fixed nitrogen may function as a stimulus to respiration or some other physiological function essential to the growth of the organisms adapted to high concentrations of fixed nitrogen.

On the addition of small amounts of fixed nitrogen growth is stimulated, thus increasing the numbers of organisms and consequently the potential fixing power of the culture. Even in the normal culture the addition of small amounts of fixed nitrogen to a "N free" medium is followed

by an immediate increase in numbers of organisms. Hills (1916) found that the addition of concentrations up to 345 p.p.m. nitrogen as KNO_3 stimulated an increase in numbers of organisms. Winogradsky (1928) observed that cultivated forms of Azotobacter react to available nitrogen with an extraordinarily abundant growth. Briscoe (1933) and others have made similar observations. Burk and Lineweaver (1930), Brown and Hart (1925), Thompson (1932), Coleman (1917), Sackett (1911), Hills (1918), and Brown and Aquino (1929), have observed that small amounts of fixed nitrogen stimulate or at least do not hinder fixation.

However, the increase in numbers with the addition of small quantities of fixed nitrogen does not explain the fact that the "4000 culture" refuses to grow appreciably on "N free" medium while the 0 culture grew abundantly. There has doubtless been some fundamental physiological alteration affected in the "4000 culture" which makes its growth dependent on available fixed nitrogen.

CONCLUSIONS

(a) Strains of Azotobacter vary considerably in their ability to tolerate relatively high concentrations of nitrate nitrogen in laboratory cultural media. This variability is apparently dependent upon the individual characteristics of the strain.

(b) Some cultures of Azotobacter, if grown at high concentrations of nitrate nitrogen for sufficient time, temporarily lose their ability to grow upon a "N free" medium that is capable of supporting abundant growth of the normal culture.

(c) Growth and nitrogen fixation by cultures which are unable to grow normally in or upon a "N free" medium are stimulated by quantities of nitrate nitrogen as small as $2\frac{1}{2}$ p.p.m.

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