THE INFLUENCE OF FIXED NITROGEN ON AZOTOBACTER

DEAN BURK AND HANS LINEWEAVER

Bureau of Chemistry and Soils, United States Department of Agriculture, Washington, D. C.

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The nitrogen-fixing organism, Azotobacter, has been little studied with reference to its normal physiological behavior in fixed nitrogen, and especially in comparison with that in free nitrogen. A knowledge of the physiology of Azotobacter growing in readily available fixed nitrogen is highly essential to controlling the interpretation of the physiological behavior of Azotobacter when fixing nitrogen, particularly in connection with ascertaining the nature of the chemical mechanism of nitrogen fixation. In accordance with this logical principle, Burk (1930) has already shown, for example, that the unique oxygen pressure functions of Azotobacter with respect to respiration, growth, nitrogen fixation, efficiency of growth, efficiency of nitrogen fixation, pH, and humic acid are qualitatively identical whether the nitrogen supply is either free or fixed, and hence that these functions do not concern directly the chemical mechanism of fixation.

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 establishing the quantitative aspects of these relations, however, previous investigators have been handicapped by the lack of a suitable manometric technique, and have employed the older chemical methods involving nitrogen and sugar analyses. Their experiments usually required several days or weeks, rather than a few hours, so that it was impossible to maintain constant, reproducible

conditions, and the ambiguous effects of old or heavy growths inevitably obtained, i.e., marked decreases in growth rate referable to mere numbers only, relative lack of nutrients circumstantially consumed, or complicated mixtures of various stages of life cycles.

It is the chief task of the present paper to analyze the normal metabolic and growth functions of Azotobacter maintained in fixed nitrogen, apart from those functions with respect to oxygen pressure which have already been considered elsewhere (Burk, 1930). The term "fixed nitrogen" will ordinarily designate "rapidly available fixed nitrogen," and will not refer to completely unavailable or slowly available nitrogen compounds.

I. METHODS

A complete description of the technique employed will be found in a previous paper (Burk, 1930). In brief, oxygen consumption has been measured according to the quantitative, physicochemical, manometric micro-methods for studying cell metabolism, well worked out and described by Otto Warburg (1926). Figures 1 and 2 show the particular type of manometer and vessel used. The vessels are filled at atmospheric pressure with various gases by passing the latter for two or three minutes down through the manometer stopcocks and out through the glass-ground necks of the side-cups, and then closing the two latter openings simultaneously. Mixtures of gases are made by means of a calibrated, multiple, all-glass flowmeter. The amounts of oxygen consumed are ordinarily too small to alter the composition of the gas appreciably. The total volume of a vessel (fig. 2) is about 16 cc., 2.00 cc. of which is occupied by the culture medium, and 0.30 cc. by the 2 N NaOH in the alkali container. Before occupying the manometer vessels, the experimental organisms are first grown in aerated gas wash bottles at 28°C. for twenty-four to forty-eight hours in a medium of the following composition: 0.8 gram K_2HPO_4, 0.2 gram KH_2PO_4 (pH 7.3); 0.2 gram $MgSO_4$; 0.2 gram NaCl; 0.1 gram $CaSO_4$; 0.01 gram $Fe_2(SO_4)_3$; 10 grams glucose; 1000 grams water. Usually such cultures are diluted somewhat with fresh medium immediately before use. The

manometers are shaken at the rate of 120 cycles per minute, with an amplitude of 3 cm.; this provides a maximum respiration rate and adequate equilibrium conditions between gas and liquid phases. The manometer readings, multiplied by a constant characteristic for each vessel, give directly the number of cubic millimeters of oxygen respired; the readings are accurate to about ± 0.5 c.mm. The growth of bacteria is measured directly by counting the number of cells before and after an experiment by means of either Hawksley-Thoma or Petroff-Hausser haemacytometers, the depths of which are 20, instead of the usual 100, microns. Before counting, the pH of a culture is brought to 5, to prevent all movement and growth. Two species of Azotobacter have been used, *A. chroococcum* Strain SM 1, obtained directly from the Rothamsted Experimental Station (England), and *A. vinelandii*, obtained originally from the New Jersey Agricultural Experiment Station.

II. THE RATIO OF GROWTH TO RESPIRATION RATE INCREASE

Whenever an inoculum is seeded into a series of vessels kept filled with gas of constant oxygen pressure, and variously treated with humic acid, ammonia, or any other reagent so that the subsequent rates of growth differ, temporal increases in the number of organisms accompany temporal increases in the rates of oxygen consumption (and likewise temporal increases of total oxygen consumption) in the same respective order. This important relation is based upon over one hundred and fifty observations. It means that by measuring the rates of respiration for merely a few hours one can determine qualitatively, and semi-quantitatively, the amount of growth and (in cases where the nitrogen supply is N_2) of nitrogen fixation.

The constant of proportionality between growth and respiration rate increase is not exactly the same for every culture of the series, however, but increases as the slope of the respiration rate time curve increases. This is true because, as will be shown, the efficiency of growth increases with the rate of growth. The relative differences between the deduced growths are therefore somewhat greater than the observed increases in respiration rate

would make them appear. In addition to the practical application mentioned above in connection with approximating growths from respiration rates, it is important in interpreting the mecha-

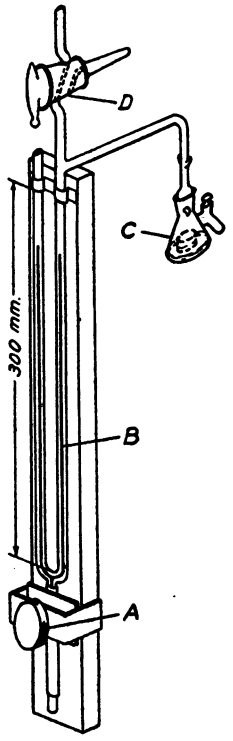

FIG. 1

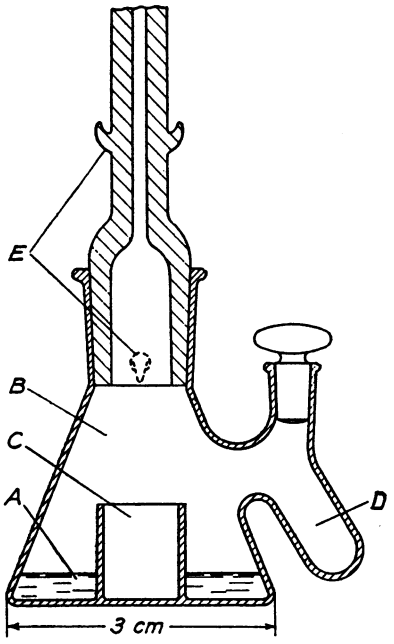

FIG. 2

FIG. 1. WARBURG-BARCROFT MANOMETER

A, screw pinchcock; *B*, manometer fluid in graduated capillary tube; *C*, respiration vessel; *D*, two-way stopcock. All glassware Pyrex.

FIG. 2. RESPIRATION VESSEL

A, culture medium (liquid phase); *B*, gaseous phase; *C*, container for CO_2-absorbing alkali; *D*, slide-cup; *E*, glass prongs for wire springs holding vessel on to manometer.

nism of fixation to determine how the constant of proportionality varies with different factors in order to ascertain whether the constants increase comparably in free and fixed nitrogen.

The constant of proportionality will be defined as

$$k = \frac{\text{final count} - \text{initial count}}{\text{initial count}} \div \frac{\text{final respiration rate} - \text{initial respiration rate}}{\text{initial respiration rate}}$$

$$= \frac{\text{growth}}{\text{initial count}} \div \frac{\text{respiration rate increase}}{\text{initial respiration rate}}$$

This constant is independent both of the units employed in measuring the count and oxygen consumption, and the amount of initial inoculum, and it approaches zero as a lower limit.

TABLE 1

k as a function of nitrogen pressure, in the presence and absence of humic acid

A. Per cent of nitrogen gas.....	0	3	10	25	45	78
B. Final respiration rate, no humic acid, 0–13 hours.....	23.8	23.9	32.5	66.3	98.8	100
C. Final respiration rate, humic acid, 0–13 hours.....	23.1	40.0	75.6	199	318	339
k { No humic acid, 0–13 hours.....	0	0	0.302*	0.288	0.467	0.696
k { Humic acid, 0–13 hours.....	0	0.347	0.596	0.635	0.662	0.850

Initial respiration rate, no humic acid, 23 c.mm. per 2 cc. per hour.
Initial respiration rate, humic acid, 25 c.mm. per 2 cc.
Initial count, 24 million per 2 cc.
1½-day old *A. chroococcum* Strain SM 1, diluted 3 times.
20 per cent oxygen, various percentages of nitrogen, hydrogen to make up to 1 atmosphere.
0.25 mgm. humic acid per 2 cc., when present.
* Slightly high.

These advantages are not possessed by the following ratios, which might otherwise have been used: (final count/initial count)/(final respiration rate/initial respiration rate); (final count − initial count)/(final respiration rate − initial respiration rate). The first of the latter two approaches 1 as a lower limit, which often results in a minimum function that is physiologically, even if not mathematically, misleading. The second is not a relative ratio, but depends upon the units employed and amounts of initial inoculum. While fairly close qualitative agreement is

TABLE 2

k as a function of concentration of humic acid

A. Milligram humic acid per 2 cc..		0	0.012	0.037	0.111	0.333	1.00
B. Final respiration rate.	0–11 hours	84.0	117	135	156	168	168
	0–14 hours	87.0	207	251	272	290	304
k.....	0–11 hours	0.435	0.515	0.625	0.745	0.785	0.785
	0–14 hours	0.780	0.900	1.07	1.25	1.31	1.25

Initial respiration rate, 15 c.mm. per 2 cc. per hour.
Initial count, 8 million per 2 cc.
1-day old *A. chroococcum* Strain SM 1, diluted 2 times.
21 per cent oxygen in nitrogen.

TABLE 3

k as a function of fixed nitrogen

A. Milligram NH_3–N per 100 cc.....		0	0.10	0.30	0.50	5.00
B. Final respiration rate.....	0– 9 hours	20.4	44.1	46.6	48.7	50.7
	0–12 hours	21.1	41.2	55.0	67.1	116
k.....	0– 9 hours	0	0.314	1.73	2.81	3.05
	0–12 hours	0	1.03	2.96	4.18	4.49

Initial respiration rate, 23 ±3 c.mm. per 2 cc. per hour.
Initial count, 22 million per 2 cc.
3-day old *A. chroococcum* Strain SM 1, diluted 3 times.
21 per cent oxygen in hydrogen.

TABLE 4

k as a function of free and fixed nitrogen, in the presence and absence of humic acid

A. Source of nitrogen.....		N_2	N_2, humic acid	NH_3–N	NH_3–N, humic acid
B. Final respiration rate.	0–10 hours	63.8	82.3	85.0	145
	0–13 hours	105	160	188	416
k.....	0–10 hours	0.658	1.70	2.81	4.15
	0–13 hours	0.643	1.53	4.94	6.25

Initial respiration rate, 8 c.mm. per 2 cc. per hour.
Initial count, 5 million per 2 cc.
1-day old *A. chroococcum* Strain SM 1, undiluted.
21 per cent oxygen in nitrogen.
0.25 mgm. of humic acid per 2 cc., when present.
0.1 mgm. of NH_3–N per 2 cc., when present.

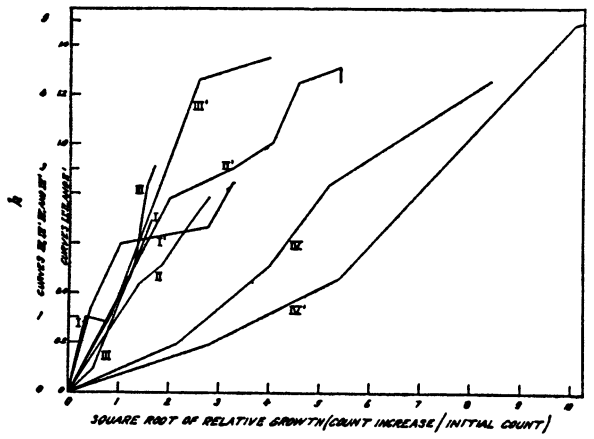

FIG. 3. INFLUENCE OF RATE OF GROWTH ON k

Curves I and I′, II and II′, III and III′, and IV and IV′ based on values of k given in, respectively, tables 1 (no humic acid and humic acid), 2 (0–11 and 0–14 hours), 3 (0–9 and 0–13 hours), and 4 (0–10 and 0–13 hours).

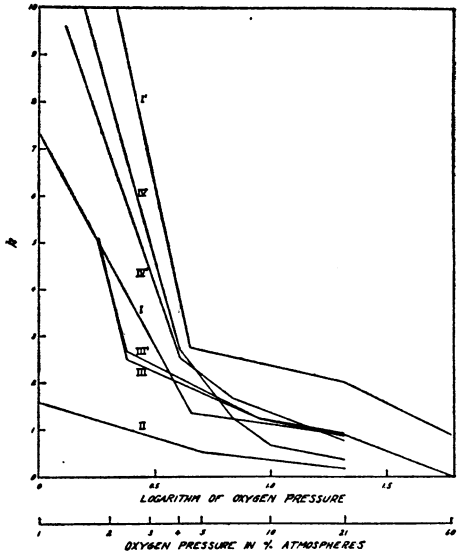

FIG. 4. k AS A FUNCTION OF OXYGEN PRESSURE

Curves I and I′, II, III and III′ and IV and IV′ based on values of k given in, respectively, tables 5 (0–8 and 0–13 hours), 6, 7 (count and nitrogen content), and 8 (count and nitrogen content).

obtained with all three constants, the first is specific and decidedly more fundamental.

It will be seen in tables 1 to 4 that k always increases with the final respiration rate. In table 1 the final respiration rate has been varied by changing the pressure of nitrogen, and by the presence of humic acid; in table 3, by changing the concentration of fixed nitrogen in the absence of free nitrogen; in table 2, by changing the concentration of humic acid; and in table 4, by the presence of humic acid and fixed nitrogen. Figure 3 (plotted as a square root function to reduce abscissal spread) shows that k also always increases with growth rate, as well as with final respiration rate; this is, indeed, the more fundamental relation, since final respiration rate is itself a function of growth, rather than *vice versa*. k does not depend solely upon rate of growth, however, since the various curves are quantitatively different. Increases of k in free and fixed nitrogen are qualitatively comparable, it should be observed.

For experiments lasting 10 ± 2 hours, k varies approximately according to the following conditions, each being at optimum.

N_2–N	½ to 1
N_2–N, humic acid	1 to 2
NH_3–N	2 to 4
NH_3–N, humic acid	4 to 6

These numerical values reflect how these conditions influence growth even more than final respiration rate, since the former is affected much more by the various conditions, than the latter. In general, humic acid doubles the rate of growth in either free or fixed nitrogen, while fixed nitrogen doubles the rate of growth as compared with that in free nitrogen.

Tables 5 to 8 and figure 4 show that when the oxygen pressure is varied, k no longer invariably increases regularly with respiration rate increase (nor, as may be merely stated, with growth), but increases rapidly as the oxygen pressure is decreased. Figure 4 has been plotted simi-logarithmically in order to condense the abscissal spread of the curves. Comparison of Curves I and I′ shows that k increases with duration and time at all oxygen pressures. Comparison of curves II and I shows that k decreases

with the age of the initial inoculum. Comparison of Curves III and III′, or IV and IV′, shows that in general little difference is noted, in the case of free nitrogen, whether k is based upon nitrogen or count determinations, at least in young, highly diluted cultures. Comparison of curves III, III′, and I shows that for inocula of the same age, and experiments of the same length, k

TABLE 5

k as a function of oxygen pressure (in fixed nitrogen)

A. Per cent of oxygen in nitrogen		60	21	4.5	1.0	0.1
B. Final respiration rate	0– 8 hours	15.0	78.9	42.6	14.0	1.3
	0–13 hours	40.0	216	113	15.0	1.3
C. Initial respiration rate (c.mm. per 2 cc. per hr.)		15.0	17.4	12.8	11.6	1.3
k	0– 8 hours	0	0.896	1.36	7.33	∞
	0–13 hours	1.40	2.01	2.75	18.1	∞

Initial count, 12 million per 2 cc.
2-day old *A. chroococcum* Strain SM 1, diluted 3 times.
0.1 mgm. NH_3–N per 2 cc.

TABLE 6

k as a function of oxygen pressure (in fixed nitrogen)

A. Per cent of oxygen in nitrogen	21	5	1	0.25	0.25 (No NH_3–N)
B. Final respiration rate, 0–8 hours	150	89	34	7.3	8.2
C. Initial respiration rate, 0–8 hours	18.2	17.1	13.9	7.3	8.2
k, 0–8 hours	0.166	0.523	1.59	∞	∞

Initial count, 50 million per 2 cc.
6-day old *A. chroococcum* Strain SM 1, diluted 7 times.
0.1 mgm. NH_3–N per 2 cc., except in case of last column, where none.

varies quite similarly in both free and fixed nitrogen. Indeed, a smooth curve may be drawn through all points of these three curves (see Burk, 1930, fig. 2, curve B, also).

Since k is defined as a direct fractional function of two other functions which increase logarithmically with time, it is inevitable that k should change with time, and, therefore, duration, when

the constants of logarithmic (or likewise geometric) increase are different for each function of the fraction, as is the case with Azotobacter. In tables 1 to 5 there is no indication, even in the experiments of longest duration, of k having reached a

TABLE 7

k as a function of oxygen pressure (in free nitrogen)

Based upon both count and nitrogen determinations

A. Per cent oxygen in nitrogen	21	9	2.4	1.8
B. Final respiration rate, 0–8 hours	44	106	33	12
C. Initial respiration rate, 0–8 hours	18	12	11	6
k (Based on count), 0–8 hours	0.865	1.23	2.50	5.12
k' (Based on nitrogen content), 0–8 hours	0.924	1.23	2.67	5.00

Initial count, 8 million per 2 cc.
Initial nitrogen, 0.6 mgm. per 2 cc. (as bacterial nitrogen).
2-day old *A. chroococcum* Strain SM 1.
k' = (nitrogen fixed/initial nitrogen content)/(respiration rate increase/initial respiration rate).

TABLE 8

k as a function of oxygen pressure (in free nitrogen)

Based upon both count and nitrogen determinations

A. Per cent oxygen in nitrogen	21	12.6	6.9	4.1	1.3
B. Final respiration rate, 0–10 hours	51	55	66	62	22
C. Initial respiration rate, 0–10 hours	24	22	22	20	15
k (Based on count), 0–10 hours	0.363	0.630	1.17	2.70	11.5
k' (Based on nitrogen content), 0–10 hours	0.741	1.33	1.67	2.54	9.62

Initial count, 18 million per 2 cc.
Initial nitrogen, 1.2 mgm. per 2 cc. (as bacterial nitrogen).
3-day old *A. chroococcum* Strain SM 1, diluted 8 times.
k' as in table 7.

maximum. The increase with time is sometimes negligible in free nitrogen, because, expressed in still another way, occasionally the second differential of relative growth is not appreciably greater than the second differential of relative respiration rate increase, with respect to time. In NH_3-N, k invariably increases with

duration. If k were defined in terms of logarithmic functions of growth and growth respiration, it would, in general, increase in a linear fashion with time.

Recapitulating, we see that although the oxidation processes of the sort involved in temporal measurements of respiration rate bear qualitative functional relationships to the frequencies of cell division, there is no very fixed quantitative ratio under widely varying conditions of oxygen concentration, nitrogen supply, age of inoculum, humic acid concentration, and time. k varies but little in the case of most aerobic microorganisms, and probably does so in the case of Azotobacter because the maximum capacity to respire is so enormously high, three times its own dry weight of glucose per hour, as compared with values only one twenty-fifth as great in the case of baker's yeast (Warburg, 1927). That is, its possible range of variation of respiration rate between small and maximum values permits k to vary more than in the case of most organisms.

III. THE CONCENTRATION OF FIXED NITROGEN REQUIRED TO INHIBIT FIXATION COMPLETELY

By growing Azotobacter in 21 per cent oxygen in, respectively, nitrogen and hydrogen, in different concentrations of rapidly available fixed nitrogen, it was found that the fractional increases in rates of respiration (final/initial rates) were the same in hydrogen and nitrogen for all concentrations of N above 0.5 mgm. per 100 cc., as shown in figure 5. Concentrations of N below this were not able to inhibit fixation completely, since growth was then greater in nitrogen than in hydrogen, i.e., nitrogen was no longer an inert gas. The values of the ratios of fractional increases in figure 5 are subject to a maximum inaccuracy of about ± 8 per cent, so that all values for concentrations of 0.5 mgm. NH_3-N per 100 cc. and above may be considered identical, within experimental error, whereas those at 0.2 and 0.1 mgm. are unquestionably higher. Other experiments described in Section IV confirm the inhibition concentration observed quantitatively. The abscissal values in figure 5 are plotted logarithmically in order to reduce spread. That the

inhibition value is independent of the concentration of bacteria in infinite dilution ranges is shown by the fact that cultures diluted three and fifteen fold give about the same ratios, over the fixed nitrogen range of concentration observed. Obviously, with very thick, heavy, old cultures, the inhibition value would be more difficult to determine, since the initial concentrations of

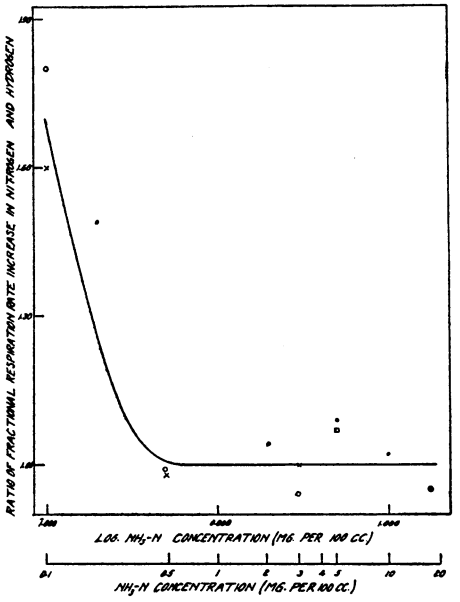

FIG. 5. FIXATION INHIBITION AS A FUNCTION OF FIXED NITROGEN CONCENTRATION

(•), the ratio (final/initial respiration rate) in N_2 and H_2, O_2 pressure being kept constant at 21 per cent in each gas. Two-day culture of *A. vinelandii*, diluted 2 times. (□) is based on NO_3-N rather than NH_3=N. 7 hours duration.

(x), and (o), the ratio (final/initial respiration) in N_2 and H_2, O_2 pressure being kept constant at 21 per cent in each gas. Three-day culture of *A. vinelandii:* (x), diluted three times; (o), diluted 15 times 6 hours duration.

fixed nitrogen added would soon decrease, and apparently higher values might be obtained.

The superiority of the present technique employed, with respect to the elucidation of fundamental characteristics of behavior, as compared with the usual extended, macro-experiments conducted without shaking to maintain equilibrium conditions, is clearly brought out. In the latter experiments, somewhat more

than the total amount of nitrogen the organisms can fix after a long period of time (one week or more) is found to be the completely inhibiting concentration, i.e., the integral rather than the differential effect is observed. The inhibiting integral concentrations so found are ten to twenty times the inhibiting differential concentrations observed by the writers. Thus, Kostyschew and coworkers (1926) found that slightly more than 15 mgm. NH_3-N per 100 cc. per gram of sugar (the amount of nitrogen the bacteria would otherwise have fixed) completely inhibited fixation. Kostyschew concluded that NH_3-N prevents fixation because (1) NH_3-N is the first product of fixation, and (2) the process is checked in accordance with the mass action law. This is hardly the case, however, as shown, indeed, by two aspects of Kostyschew's data, which the present writers themselves have confirmed. (1) The quantitative inhibition relations observed in NH_3-N are the same as in NO_3-N, a form of nitrogen which, according to Kostyschew, is not concerned in the mechanism, and therefore, presumably, not capable of mass action law effect against fixation. (2) The integral inhibiting concentration is too closely identical with the quantitative nitrogen requirements to mean other than that, as shown by figure 5, and numerous workers previously, the integral and differential limiting concentrations are determined solely by the nutritional needs of the bacteria as determined, in turn, by the capacity to divide and grow under the particular conditions of the environment. In this connection, rates as well as amounts must be considered. Thus, Kostyschew found that in the case of peptone nutrition even 130 mgm. of N per 100 cc. did not entirely prevent fixation; here, however, the nitrogen is much less rapidly available than in the case of NH_3-N or NO_3-N. Zoond (1926), incidentally, found a very much smaller amount of peptone, about one-fifteenth, to be completely inhibiting.

IV. GROWTH AS A FUNCTION OF FIXED NITROGEN CONCENTRATION

The rate of growth (ratio of final/initial respiration rate) as a function of rapidly available fixed nitrogen passes through a maximum at about 0.5 to 1 mgm. N per 100 cc., falling off sharply

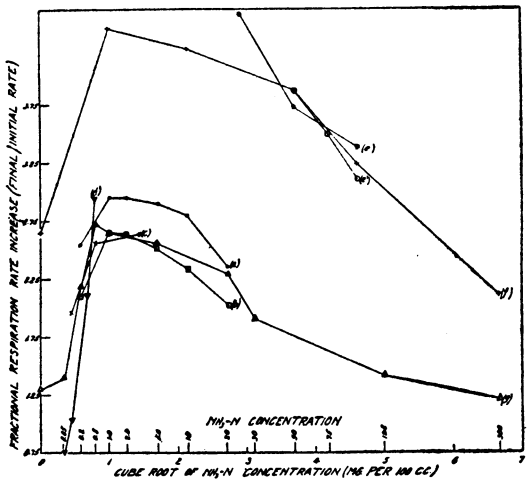

FIG. 6. GROWTH AS A FUNCTION OF FIXED NITROGEN

a (•), 2 day culture, diluted 2 times, 7 hours duration, 21 per cent O_2 in N_2. (x), NO_3-N instead of NH_3-N.
b (□), 2 day culture, diluted 2 times, 6 hours duration, 21 per cent O_2 in H_2.
c (x), 3 day culture, diluted 2 times, 6 hours duration, 21 per cent O_2 in H_2.
d (▽), 2 day culture, diluted 0 times, 24 hours duration, 21 per cent O_2 in H_2.
e (*), 1 day culture, diluted 2 times, 8 hours duration, 21 per cent O_2 in N_2. (e′, (o), NO_3-N instead of NH_3-N.)
f (+), 2 day culture, diluted 2 times, 8 hours duration, 21 per cent O_2 in N_2.
g (△), 3 day culture, diluted 1½ times, 7 hours duration, 21 per cent O_2 in N_2.
A. vinelandii used in all cultures.

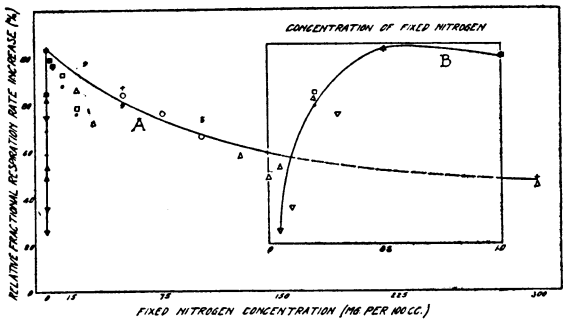

FIG. 7. GROWTH AS A FUNCTION OF FIXED NITROGEN

Curve A, replot of all curves of figure 6, on a relative percentage basis, as a direct rather than cube root function of concentration.
Curve B, same type of plot as in curve A, drawn to large scale to show abscissal portion of concentration between 0 and 1 mgm. N per 100 cc.
Points and experiments as in figure 6.

TABLE 9

Total respiration as a function of fixed nitrogen

Experiment a:										
Concentration of NH_3–N (mgm. per 100 cc.).....	0	0.05	0.2	0.5	1	5	20	30	125	300
Total respiration (c.mm. of O_2 per 8 hours).....	397	438	466	570	550	548	512	418	404	318
Experiment b:										
(Concentration of NH_3–N (mgm. per 100 cc.).....	0	1	25	50	100	300				
Total respiration (c.mm. of of O_2 per 8 hours).....	666	937	873	815	678	451				
Experiment c:										
(Concentration of NH_3–N (mgm. per 100 cc.).....	0.2	1	2	10	20					
Total respiration (c.mm. of O_2 per 7 hours).....	964	1109	1071	1066	1036					
Experiment d:										
Concentration of NH_3–N (mgm. per 100 cc.).....	0	0.05	0.1	0.3	0.5					
Total respiration (c.mm. of O_2 per 54 hours).....	31	66	81	140	232					
Experiment e:										
Concentration of NH_3–N (mgm. per 100 cc.).....	0	0.05	0.1	0.3	0.5					
Total respiration (c.mm. of O_2 per 54 hours).....	0	61	73	132	190					
Experiment f:										
Concentration of NO_3–N (mgm. per 100 cc.).....	0	0.5	2.5	5	10					
Total respiration (c.mm. of O_2 per 7 hours).....	465	700	690	700	712					
Experiment g:										
Concentration of NH_3–N (mgm. per 100 cc.).....	0.1	0.5	3.0							
Total respiration (c.mm. of O_2 per 6 hours).....	397	500	508							

a. 3-day culture diluted 1¼ times, 21 per cent O_2 in N_2.
b. 2-day culture diluted 2 times, 21 per cent O_2 in N_2.
c. 2-day culture diluted 2 times, 21 per cent O_2 in H_2.
d. 2-day culture diluted 0 times, 21 per cent O_2 in N_2.
e. 2-day culture diluted 0 times, 21 per cent O_2 in H_2.
f. 2-day culture diluted 2 times, 21 per cent O_2 in H_2.
g. 3-day culture diluted 3 times, 21 per cent O_2 in N_2.
All cultures *A. vinelandii*.

at lower concentrations, but less rapidly at higher concentrations, as shown in either figures 6 and 7, based upon respiration rate increases, or table 9, based upon total respiration. The abscissal values in figure 6 are plotted as cube root functions in order to reduce spread; the fall in rate at higher concentrations is therefore much slower, and at lower concentrations, much higher, relatively, than would appear at first glance (see curve A, fig. 7).

The rate of growth between 0.5 and 10 mgm. N per 100 cc. is, for practical purposes, identical, the decrease at the latter concentration being only a few per cent (see especially experiment *f*, table 9, and curves a, b, g, fig. 6). Experiment *f*, table 9, also shows that maximum growth is reached by at least 0.5 mgm. NO_3-N as well as by 0.5 mgm. NH_3-N per 100 cc. Curve *e'*, figure 5, shows that high concentrations of NO_3-N cause decreases in rates of growth just as do high concentrations of NH_3-N, as in curve *e*, so that the inhibition is owing to N as such, rather than to the nature of the chemical compound in which it appears. The pH was, of course, always constant in these experiments. It is interesting to note that growth in 200 to 300 mgm. N per 100 cc. is less than in free nitrogen; this observation has been confirmed by count measurements also.

The fact that maximum growth occurs at about 0.5 mgm. N per 100 cc. confirms the previous finding that the same, or at least no greater, concentration causes complete inhibition of nitrogen fixation, and, moreover, supports the view that the inhibiting effect of higher concentrations is not based upon mass law action, but simply upon the maximum metabolic capacity for using nitrogen, whether free or fixed. Growth is, indeed, considerably greater at 0.5 mgm. fixed nitrogen per 100 cc. (see *k* values in Section II) than in free nitrogen, so that obviously, in agreement with the view of Bonazzi (1924) and many others, nitrogen fixation is a means of nitrogen nutrition resorted to only in cases of relative nitrogen starvation.

That fixed nitrogen (either as $(NH_4)_2HPO_4$ or KNO_3) at no very great concentrations should cause decreases in the rate of division and the rate of its own utilization, is by no means easily understood, especially since the effect is independent of the kind

of rapidly available fixed nitrogen, the nature of the charge of the ion bearing the nitrogen, obvious antagonism effects, or general osmotic effects. Burk (1930) has given two general type explanations, based upon chain reaction kinetics and contact catalysis, of the similar case of inhibition of oxygen consumption by high oxygen pressures, which might likewise apply here in principle, although hardly in detail.

TABLE 9a

The nitrogen content of azotobacter supplied free nitrogen and different amounts of fixed nitrogen

Experiment 1:						
NH_3—N added (mgm. per 100 cc.).....	2	2	4	4	6	6
Dry matter obtained (mgm. per 100 cc.)....	45.3	48.3	54.2	56.0	69.3	77.3
Total nitrogen obtained (mgm. per 100 cc.).	5.00	5.24	6.35	6.47	7.80	7.93
Per cent nitrogen in dry matter.....	11.0	10.8	11.7	11.6	11.3	10.3
Experiment 2:						
NH_3—N added (mgm. per 100 cc.).....	2	3	4	4	5	
Dry matter obtained (mgm. per 100 cc.)....	44.4	52.8	60.7	59.6	70.4	
Total nitrogen obtained (mgm. per 100 cc.).	3.55	4.31	5.22	5.22	6.08	
Per cent nitrogen in dry matter.....	7.9	8.2	8.6	8.8	8.6	

All experiments with *A. chroococcum* Strain SM 1, grown in 1-liter Roux flasks in air thermostat at 28°C. 75 cc. culture medium per flask, filling flask to depth of 3 mm. 1 drop of inoculum of young culture used. Initial concentration of sugar 1 per cent; sugar in no case entirely consumed. The added NH_3—N was in all cases consumed before end of experiment. Nitrogen determined by Pregl microkjeldahl method, dry matter by centrifuging 20 cc. portions of culture, decanting supernatant fluid, drying precipitate *in vacuo* at room temperature and weighing to the fourth decimal place.

Experiment 1, culture grown for 10 days.

Experiment 2, culture grown for 6 days.

The view that nitrogen fixation is determined by the metabolic capacity for using nitrogen whether free or fixed is further supported by the data given in table 9a, based upon a different technique. It will be seen that in spite of the different amounts of nitrogen supplied (and the consequently different amounts of growth occurring), the nitrogen content of the dry matter is practically constant, i.e., the organisms have fixed nitrogen only so far as their normal needs require.

It has already been shown (Burk, 1930) that although humic

acid greatly stimulates the observed rate of nitrogen fixation, its action is not upon the process of fixation directly, but merely upon cell division or growth, the velocity of which to some extent determines the rate of fixation. The growth of organisms in fixed nitrogen is equally subject to stimulation by humic acid, as are also the other functions of initial respiration rate, final respiration rate, total respiration, growth, and efficiency of growth.

V. TOTAL RESPIRATION, GROWTH, AND EFFICIENCY OF GROWTH AS FUNCTIONS OF LIMITING CONCENTRATIONS OF FIXED NITROGEN

It has been found that with infinite dilutions of Azotobacter (0 to 200 million per cubic centimeter) the maximum rate of growth is reached at concentrations of 0.5 to 1 mgm. NH_3-N per 100 cc. Corresponding growth efficiency measurements were therefore determined from this approximate region of concentration down to zero concentration. A three-day old culture of *A. chroococcum* Strain SM 1, diluted 3 times with inorganic nutrient solution, made up to 0.5 per cent glucose, and containing 22 million bacteria per 2 cc., was grown in 6 different concentrations of NH_3-N in a gas containing 10 per cent O_2 in H_2, for nine and twelve hours respectively (twelve simultaneous sub-experiments in all). Air as a source of oxygen was not used, in order to avoid the complicating effects of nitrogen fixation by organisms growing in markedly limiting concentrations of NH_3-N, and 10 per cent O_2 was employed as a compromise between the normal value, 21 per cent, and 5 per cent, where growth (in NH_3-N as well as N_2-N) is at a maximum with respect to oxygen concentration.

Table 10 gives the experimental results and table 11 the efficiency calculations. The results for 9 to 12 hours were obtained by subtracting those at 0 to 9 hours from those at 0 to 12 hours, making virtually three different experiments in all. The weighted average values have always been determined by giving double weight to 0 to 12 hours, where the experimental accuracy is highest. The observed total respiration, which is composed of respiration by both the new growth and the initial inoculum, is corrected for the latter by subtracting the total respiration

observed at 0.00 mgm. NH_3-N per 100 cc., the control in which no growth occurred. Efficiency calculations based upon respiration due to growth only (growth respiration) are the more fundamental and absolute numerically, although qualitatively the uncorrected respiration values lead to identical conclusions. The

TABLE 10

Growth and respiration as functions of limiting concentrations of fixed nitrogen (experimental data)

Concentration of NH_3-N (mgm. 100 cc.)	0.00	0.02	0.10	0.30	0.50	5.0
Count increase (millions per 2 cc.) (initial = 22 millions per 2 cc.):						
0– 9 hours	0	2	6	38	54	66
0–12 hours	0	4	16	78	146	358
9–12 hours	0	2	10	40	92	292
Weighted average	0	3.0	12.0	58.4	109.6	268
Total respiration (c.mm. O_2 per 2 cc.):						
0– 9 hours	184 (193)	238 (240)	300 (287)	294 (296)	312 (306)	302 (307)
0–12 hours	261	325	414	466	493	585
9–12 hours	77	87	114	172	181	283
Weighted average	196	244	310	349	370	414
Growth respiration (respiration due to growth) (c.mm. O_2 per 2 cc.):						
0– 9 hours	0	54	116	110	128	118
0–12 hours	0	64	153	205	232	324
9–12 hours	0	10	37	95	104	206
Weighted average	0	48	115	154	174	243

method of correction used in the present experiments is exact, since, (1) no growth occurred in the control, and (2) the respiration rate per hour in the control remained constant during the whole experiment, indicating complete freedom from abnormal behavior. In evaluating the results, it must be observed that the experimental inaccuracy was about ± 4 per cent in the most

significant and instructive range (0.1 to 0.5 mg. NH_3-N), but very much larger for 0.02 mgm. NH_3-N.

Table 10 shows the continous increase of count, total respiration, and total respiration increase, with NH_3-N concentration and time. The figures in parenthesis for total respiration, 0 to 9 hours, are those of the simultaneously grown 0 to 12 hour cultures, and are given to indicate the degree of duplication obtained. The agreements at the different increasing concentrations of NH_3-N agree to within, respectively, 4.9, 0.8, 4.3, 0.6, 2, and 1.6, or an average of, 2.4 per cent.

TABLE 11

Efficiencies of growth as functions of limiting concentrations of fixed nitrogen (calculated data)

Concentration of NH_3-N (mgm. per 100 cc.)...	0.00	0.02	0.10	0.30	0.50	5.00
Count increase / Total respiration:						
0- 9 hours	0	0.008	0.020	0.130	0.172	0.222
0–12 hours	0	0.012	0.038	0.168	0.296	0.612
9–12 hours	0	0.024	0.088	0.234	0.510	1.032
Weighted average	0	0.014	0.046	0.176	0.318	0.620
Count increase / Growth respiration:						
0- 9 hours	0	0.036	0.052	0.346	0.422	0.560
0–12 hours	0	0.062	0.104	0.380	0.630	1.106
9–12 hours	0	0.200	0.272	0.422	0.886	1.418
Weighted average	0	0.090	0.132	0.382	0.642	1.048

Table 11 shows the continuous increase of efficiency (cell increase in millions per 2 cc./respiration in cubic millimeters per 2 cc.) with concentration of NH_3-N and time, for all concentrations. The magnitude of the effect is indeed striking, a 10 to 20 fold increase occurring between 0.02 and 5 mgm. NH_3-N in the case of the growth respiration efficiency, and a 25 to 50 fold increase occurring in the case of the total respiration efficiency.

The straight line function of efficiency of growth with respect to concentration of rapidly available fixed nitrogen, whether based upon total or growth respiration, is shown in figure 8,

curves II and I, respectively, using weighted average efficiencies for the three experiments in each case. Smoothed straight lines may be drawn for 0 to 9, 0 to 12, and 9 to 12 hour experiment efficiencies, as well as for their unweighted average, the least deviation of points from the line being obtained at 0 to 12 hours, where the experimental error is least.

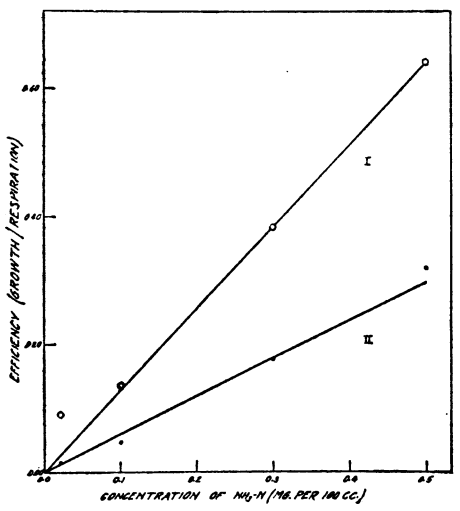

FIG. 8. EFFICIENCIES AS A FUNCTION OF LIMITING CONCENTRATIONS OF FIXED NITROGEN AND GROWTH

Curve I, weighted average efficiency, based upon respiration due to growth only, plotted against NH_3-N concentration.
Curve II, weighted average efficiency, based upon total respiration (i.e., including respiration of inoculum also), plotted against NH_3-N concentration.
Curve III, weighted average efficiency, based upon growth respiration, plotted against growth (count increase).

Curve I, figure 9, a replot of curve II, figure 8, to a larger scale, shows that maximum efficiency with respect to NH_3-N concentration is obtained at the same point on the abscissa where maximum growth is obtained, i.e., 0.5 to 1 mgm. NH_3-N per 100 cc. Curve II, figure 9, a plot of weighted average growth against NH_3-N concentration, shows that the relation is also a straight line up to nearly maximum growth. The quantitative ordinate differences between efficiencies (and likewise growths)

at concentrations of 0.5 and 5 mgm. NH_3-N are in some degree apparent, since the relative decrease in concentration from the initial has been greater in the former.

Figure 10 shows the extremely important fact that the efficiency of growth depends markedly upon the rate of growth. The relation is not quite linear, but is slightly concave downward.

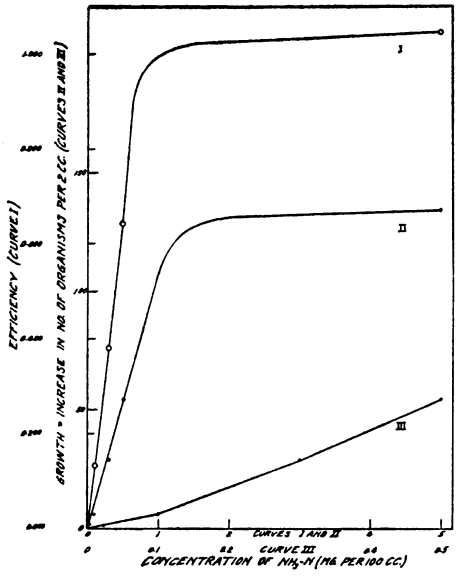

FIG. 9. EFFICIENCY AND GROWTH AS FUNCTIONS OF LIMITING CONCENTRATIONS OF FIXED NITROGEN

Curve I, replot of curve I, figure 1, on smaller scale.
Curve II, weighted average growth plotted against NH_3-N concentration.
Curve III, weighted average growth plotted against NH_3-N concentration, on larger scale.

This is the case, also, when growth is governed by limiting pressures of free nitrogen, even though growth when so governed is itself linear with respect to nitrogen pressure (Burk, 1930), just as growth in fixed nitrogen is linear with respect to limiting concentrations of fixed nitrogen.

Growth respiration, in distinction to growth and efficiency, does not vary with fixed nitrogen concentration directly, but as

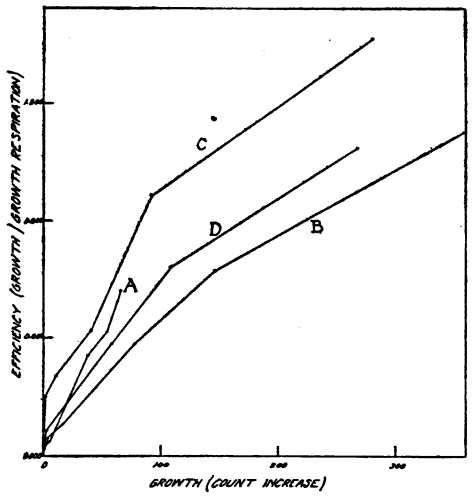

FIG. 10. EFFICIENCY OF GROWTH AS A FUNCTION OF GROWTH

Curves A, B, C, D, efficiencies based upon growth respirations at, respectively, 0–9 hours, 0–12 hours, 9–12 hours, and for weighted average values (see table 11).

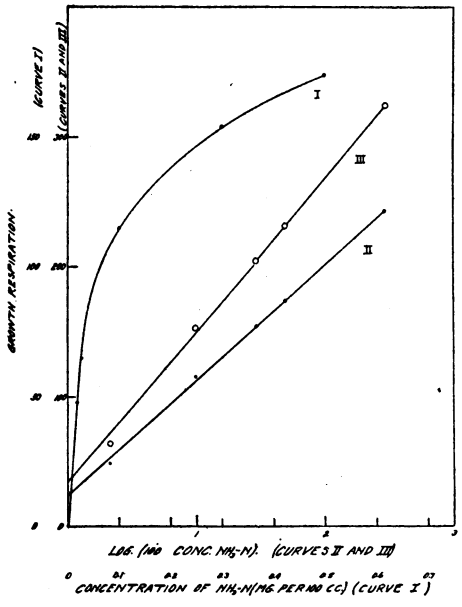

FIG. 11. GROWTH RESPIRATION AS A FUNCTION OF LIMITING CONCENTRATIONS OF FIXED NITROGEN

Curve I, weighted average growth respiration plotted directly against NH_3-N concentration.

Curve II, weighted average growth respiration plotted against logarithm of NH_3-N concentration.

Curve III, growth respiration, 0–12 hours, plotted against logarithm of NH_3-N concentration.

its logarithm. This is shown in figure 11, curves II and III, plots of, respectively, weighted average growth respiration and 0 to 12 hour growth respiration against logarithm (100 NH_3-N concentration). Curve I shows weighted average growth respiration plotted directly, rather then logarithmically, with respect to NH_3-N concentration.

The physiological characteristics of growth behavior of Azotobacter maintained in limiting, non-inhibiting, concentrations of rapidly available fixed nitrogen may be summarized analytically as follows. If, for any given duration of time, x is the growth (in millions of bacteria per 2 cc.), and y the growth respiration (in cubic millimeters of oxygen per 2 cc.), when N is the concentration of NH_3-N (in mgm. per 100 cc.) then

$$x = a\,N \qquad \text{(Fig. 9)} \qquad (1)$$

$$x/y = b\,N \qquad \text{(Fig. 8)} \qquad (2)$$

$$d(x/y)/dx > 0;\ d^2(x/y)/dx^2 < 0 \qquad \text{(Fig. 10)} \qquad (3)$$

$$y = c \ln N + d \qquad \text{(Fig. 11)} \qquad (4)$$

where a, b, c, and d are constants, which depend, incidentally, chiefly upon the duration of the experiment, i.e., time. Equation (2) could not hold if equations (1) and (4) are valid, were it not for the fact that $d(x/x^\circ)/d\mathrm{N}$ is very much greater than $d(y/y^\circ)/d\mathrm{N}$ (where x° and y° are the count and respiration, respectively, when $\mathrm{N} = 0$), so that the latter is practically (i.e., experimentally) a constant with respect to the former. This may be seen from table 10 where between concentrations of 0.02 and 5 mgm. N the weighted average growth increases 89 fold (268/3) whereas the weighted average growth respiration increases only 5 fold (243/48), or a relative ratio of 18 fold (89/5). This, in itself, is a remarkable circumstance, and expresses, in still another way, after the manner of variation of both k and efficiency of growth with rate of growth, that, in Azotobacter, growth and growth respiration are by no means parallel functions.

If smoothed curves of efficiencies of growth are plotted against NH_3-N concentration for each of the three experiments (rather than for their weighted averages in figure 8, curve I), the values of b in Equation (2) are found to increase somewhat with time, that is, $d((x/y)/d\mathrm{N})/dt > 0$, where t is time. So far as can be

determined, $d^2((x/y)/d\mathrm{N})/dt^2$ is zero. Also, as is very obvious from inspection of table 11, for any given NH_3-N concentration the efficiency increases with time, that is, $d(x/y)/dt > 0$. As pointed out before, when considering k, in fixed nitrogen the second differential of fractional growth is always greater than the accompanying second differential of fractional growth respiration.

The equally marked dependence of efficiency of growth upon oxygen pressure, increasing ten to twenty fold between 0.21 and 0.001 atmosphere, whether the organisms are grown in either free or fixed nitrogen, is to be clearly distinguished from its dependence upon rate of growth at constant oxygen pressure. In the range of oxygen pressure where the efficiency of growth is increasing markedly, that is, below, 0.05 atmosphere, the growth rate is actually decreasing. In this range, the rate of growth respiration is decreasing much faster than the rate of growth, which of course, accounts for the efficiency itself increasing. One can perhaps picture the oxygen pressure effect on efficiency as concerned chiefly with the respiration enzyme, possibly in the region of the cell wall, the growth rate effect on efficiency, however, exerting its influence inside the cell at the locus where the mechanism of division takes place. The very great bearing of the relationship of the rate of growth to efficiency of growth upon the energetics of nitrogen fixation will be considered elsewhere.

SUMMARY

1. 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 cc.

2. The rate of growth as a function of concentration of rapidly available fixed nitrogen passes through a maximum at a concentration of 0.5 to 1 mgm. per 100 cc., falling off sharply at lower concentrations, but much less rapidly at higher concentrations.

3. The efficiency of growth in fixed nitrogen depends markedly upon the rate of growth. Hence, although increases in rates of respiration with time may be used as a qualitative measure of

the amounts of growth occurring simultaneously, the constant of proportionality, k, between growth and growth respiration rate increase increases with the rate of growth, which in turn may depend upon pressure of nitrogen gas, concentration of fixed nitrogen, presence of humic acid, oxygen concentration, age of inoculum, and time.

4. The percentage composition of nitrogen in the dry matter of the cells varies little whether the nitrogen supply is chiefly fixed or free.

5. The view of previous workers is supported and enlarged upon, that fixation is a function resorted to only in the absence of sufficiently available fixed nitrogen.

6. The various physiological functions of respiration, growth, efficiency, etc., of Azotobacter maintained in free and fixed nitrogen have been compared qualitatively. The behavior is quite similar in the two cases, and no conclusions may be drawn, therefore, concerning the chemical mechanism of nitrogen fixation.

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