THE EFFECT OF OXYGEN ON NITROGEN FIXATION BY *AZOTOBACTER*

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SUMMARY

Measurements of nitrogen fixation in *Azotobacter vinelandii* plotted on a LINEWEAVER-BURK plot at oxygen concentrations of 10 and 20 % indicate a competitive inhibition of nitrogen fixation by oxygen. At higher oxygen concentrations there was an additional inhibition of respiration and growth. The Michaelis constants for nitrogen fixation were 0.0107 and 0.0229 at 10 and 20 % oxygen respectively. It is suggested that oxygen and nitrogen may compete as terminal hydrogen acceptors, nitrogen fixation thus being a form of respiration.

INTRODUCTION

In an aerobic nitrogen fixing organism, such as *Azotobacter*, respiration and nitrogen fixation may be considered alternative pathways of oxidation, the hydrogen of reduced substrates being transferred ultimately in the first case to oxygen and in the second to nitrogen. If any of the same hydrogen donors are concerned in these two pathways, then, under conditions where supply of hydrogen donors is a limiting factor, we might expect to find oxygen and nitrogen competing for such donors and hence oxygen inhibiting nitrogen fixation.

Inhibition of nitrogen fixation by oxygen was first reported by MEYERHOF AND BURK[1], but this was later considered by BURK[2] to be a general inhibition of growth rather than a specific effect on nitrogen fixation, since he found a similar inhibition of growth of the organism when supplied with fixed nitrogen. PARKER[3] and TSCHAPEK AND GIAMBIAGI[4] also found inhibition of nitrogen fixation by oxygen but did not determine whether the effect was specific or due to a general depression of growth caused by inhibition of some other system.

HAMILTON AND WILSON[5] found that *Aerobacter aerogenes* could fix nitrogen under anaerobic but not under aerobic conditions, and the specific nature of the inhibition of nitrogen fixation by oxygen was shown later for this organism[6] and for a *Bacillus* of the *B. polymyxa* type[7].

This paper reports further examination of the quantitative effect of oxygen on nitrogen fixation and investigation of the nature of the inhibition by the method of LINEWEAVER AND BURK[8]. *Azotobacter vinelandii* was used as the test organism.

A preliminary note of this work has been published[9].

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MATERIALS AND METHODS

Organism

The strain of *Azotobacter vinelandii* used was kindly supplied by Dr. R. J. SWABY of the C.S.I.R.O. Division of Soils. It had several advantages for work of this kind. It was strictly aerobic and displayed no tendency to fermentation reactions at low oxygen partial pressures; it metabolised glucose or sucrose very actively; it did not alter the pH of the medium; and in suitable gas mixtures it had a generation time of about 1.5 h, even in the rigidly defined test media in which it was grown.

One serious disadvantage found was a tendency of the cells to agglutinate when grown in Warburg vessels. The clumps of cells varied in size from a few cells to several hundreds. No way was known of calculating how much oxygen was reaching the inner cells in the clumps, and it was observed that oxygen uptake usually showed a good deal of variation when agglutination was pronounced.

Attempts at obtaining "non-agglutinating" cultures by selection from the original strain met with some success, but these would revert rapidly to the agglutinating form. The same effect was found with other strains of *A. vinelandii* and *A. chroococcum* tested.

This problem was solved by centrifuging the culture lightly (about 1000 $\times$ *g* for 5 to 10 min) when it was found that the cells remaining suspended gave a culture which would grow for at least 6 h without agglutination. The oxygen consumption of such cells was found to be similar to that of the original culture but less variable.

Preparation of media

All reagents used were A.R. grade. The liquid medium was dispensed in 30–50-ml portions in 4 ounce McCartney bottles with glass caps; solid media were dispensed in 12–15-ml portions in 1 ounce McCartney bottles. These were then sterilised at 5 lbs. pressure for 30 min.

A nitrogen-free medium (M22) suitable for turbidimetric work was developed, having the following composition: sucrose 10 g, Na_2HPO_4 150 mg, K_2HPO_4 150 mg, $MgSO_4 \cdot 7H_2O$ 200 mg, $CaSO_4$ 30 mg, NaCl 100 mg, Na_2MoO_4 3 mg, $FeSO_4 \cdot 7H_2O$ 3 mg, $MnSO_4 \cdot 4H_2O$ 2 mg, $CuSO_4 \cdot 5H_2O$ 0.4 mg, $ZnSO_4 \cdot 7H_2O$ 0.8 mg, H_3BO_3 2.5 mg, ethylene diamine tetra-acetic acid Fe salt 10 mg, cysteine 3 mg, glass distilled water to 1 l. The pH was adjusted to 7.5, when necessary.

To make solid medium 2 % of Davis Bacteriological agar was added.

With the very low culture densities used in this work the rates of growth in this medium were superior to those usually reported by other workers. The phosphate content was kept low since it was found that growth was inhibited by phosphate at the levels used in BURK's[10] medium. The organism did not affect the pH of the medium under the conditions used.

Gas mixtures

Gases were mixed and stored over saturated sodium chloride solution. Argon was used as the inert diluent gas, as a product of very high purity (British Oxygen Co., 99.8 % pure) was available.

Gas concentrations are referred to throughout as percentages of one atmosphere.

Method of culture

Cultures were grown in Warburg respirometer vessels at 30°, this being a convenient method of providing a controlled atmosphere and temperature. The cultures were grown overnight with aeration, starting from an inoculum adjusted so as to ensure that the cells would be still in the logarithmic growth phase in the morning. They were then suitably diluted and aerated for 1.5 to 2 h to eliminate any lag period at the beginning of an experiment. If necessary a further slight dilution was made and 1-ml or 2-ml aliquots were transferred to Warburg flasks, of about 25 ml total gas volume with 0.1 ml of 20 % potassium hydroxide absorbed on filter paper rolls in the centre wells.

The required atmosphere was provided by thrice evacuating the flasks and refilling from a reservoir of the gas mixture[11], and all experiments were performed at atmospheric pressure.

Samples were taken at the beginning and end of the experimental period for nitrogen analyses.

Nitrogen analysis

Nitrogen analyses were carried out by an improved ultra micro Kjeldahl method[12].

Turbidimetric measurements

For preliminary estimation of the dilution required the O.D. of cultures was measured at 600 mμ in a Unicam S.P. 600 spectrophotometer in 5-mm cells. It was found that for O.D. below 0.1 there was a linear relation between amount of culture and O.D.

Optical measurements were not used as a precise measure of growth, since it was found that even in the complete absence of any source of nitrogen, free or fixed, the O.D. of a culture might increase by up to 100 % in 3 h. This may have been due to more effective use of nitrogen from parent cells by daughter cells, or to production of carbohydrate gums or other materials.

Manometric measurements

For respiration measurements the oxygen uptake was measured at 15-min intervals. The shaking rate was 107/min.

Oxygen uptake was not used as a measure of fixation as it was found that there was not a good correlation between increase in oxygen uptake and increase in fixed nitrogen[13]. The oxygen uptake however did give a useful indication of rate of growth and a means of ensuring that the right oxygen concentration was being maintained. To ensure this it was necessary to work with dilute cultures. The relation of oxygen uptake to amount of culture was found to be linear up to 400 μl/h at 20 % oxygen and up to 100 μl/h at 5 % oxygen. In order to maintain the number of cells well below the point where rate of diffusion of gas could be a limiting factor it was necessary to start from cultures with an uptake of about 80 μl/h in 20 % oxygen. Such a sample would contain 3–4 μg of nitrogen, show an O.D. of 0.02–0.03 in a 5-mm cell and give a count of about $15 \cdot 10^6$ *Azotobacter* cells by the method of GALL, STARK AND LOOSLI[14].

Glassware

With such dilute cultures in a synthetic medium traces of impurities represent

a serious source of experimental variability. In particular it was found that arsenic (present in appreciable amount in the borosilicate glass used) can have a considerable effect on cultures, 0.1 μg/ml causing 20–30 % reduction in respiration rate. Variability from this or other impurities was overcome by autoclaving the vessels in 1 % nitric acid and rinsing in double glass distilled water.

RESULTS

Effect of oxygen on respiration

The effect of atmospheres varying in concentration from 5 to 100 % oxygen in argon on the oxygen uptake of *Azotobacter vinelandii* is shown in Fig. 1. There is a certain variability in the rate of respiration and the shape of the curve obtained for different subcultures, but all show a maximum at 25–30 % oxygen.

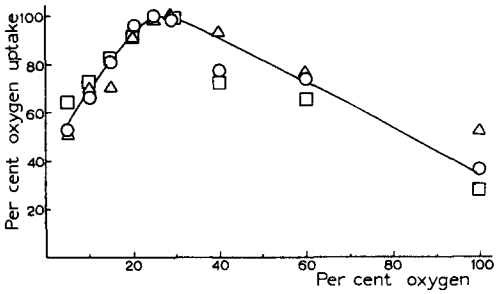

Fig. 1. Oxygen uptakes of *Azotobacter* at different concentrations of oxygen in argon expressed as per cent of the maximum uptake. Circles, triangles and squares represent results from three different subcultures.

This differs from the maximum found by BURK[2] at about 15 % oxygen. This difference could possibly be due to the different methods of preparation of the culture. Ours was grown with aeration overnight in nitrogen-free medium, so that there may have been a tendency to select oxygen resistant cells. BURK's cultures were usually 2–3 days old still cultures, diluted to give suitable oxygen consumption in the Warburg flasks, and may thus have been adapted to a lower oxygen tension. That the response to oxygen concentration is markedly affected by the atmosphere to which the culture has adapted is shown in Fig. 2. This represents the respiration at different oxygen

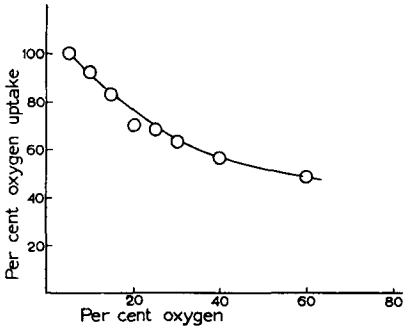

Fig. 2. Oxygen uptake at different concentrations of oxygen in argon of *Azotobacter* previously grown at 2% oxygen. Uptake as per cent of the maximum.

tensions of a culture grown on M22 agar at 2 % oxygen in nitrogen for three transfers, then overnight in M22 broth gassed with the same atmosphere, and then diluted and measured. The maximum respiration was at 5 % oxygen or less, indicating a high degree of adaptation to the atmosphere in which it grew, although the rate of respiration was only about 60 % of that of a normal culture.

The respiratory quotient was measured for oxygen concentrations of 5–100 % and found to be 0.98 ± 0.04. There was no indication of any change in the pH of the medium. Thus it would appear that, as BURK[2] concluded, *Azotobacter* completely oxidizes glucose to carbon dioxide and water.

Effect of oxygen on nitrogen fixation

The effect of oxygen concentration on nitrogen fixation was studied at oxygen concentrations 10, 20, 30 % and nitrogen 2, 4, 8, 16 %. The remainder of the atmosphere was argon giving a final pressure of 1 atm in each case.

5 % oxygen was not used, as, in order to avoid an oxygen deficiency after 3 h growth, it would be necessary to use an initial culture density too low for accurate measurement. Even at 10 % oxygen after 3 h growth the rate of oxygen uptake frequently approached the permissible maximum.

The range of each experiment was limited by the size of the Warburg bath. Thus it was possible to measure the effect of only two concentrations of oxygen at four different levels of nitrogen in the one experiment. Comparison between different experiments was uncertain, due to variability between subcultures.

Table I shows the results of eight experiments in which nitrogen fixation was measured at 10 % and 20 % oxygen.

Assuming nitrogen fixation to follow an exponential law the rate can be expressed as the velocity constant, k, defined by $dN/dt = kN$ where N is the fixed nitrogen concentration and t is the time (in hours). Then

$$k = \frac{1}{t} \ln \frac{\text{nitrogen at time } t}{\text{initial nitrogen}}$$

Table II shows the k values thus obtained from the data of Table I.

The value of k is thus a measure of rate of nitrogen fixation under different

TABLE I

TOTAL BACTERIAL N IN μg IN 1-ml CULTURE INITIALLY AND AFTER GROWTH IN WARBURG FLASKS

Growth for 3 h at varying oxygen and nitrogen pressures.

Expt. no.	*Initial nitrogen*	*Final nitrogen*							
		20% oxygen Nitrogen per cent				*10% oxygen Nitrogen per cent*			
		16	*8*	*4*	*2*	*16*	*8*	*4*	*2*
1	3.62	8.9	8.20	6.46	5.62	9.73	9.66	—	7.43
2	4.00	10.0	9.1	8.0	—	11.6	10.3	9.7	8.6
3	3.70	10.0	9.6	9.0	7.1	12.0	10.5	—	9.0
4	3.20	9.1	8.2	7.4	5.5	10.1	9.2	8.2	7.6
5	3.20	9.3	7.6	6.9	—	10.0	9.3	7.6	6.9
6	2.90	9.8	7.2	6.0	5.0	—	8.5	7.4	5.7
7	3.40	9.7	7.6	6.6	6.0	10.6	8.8	—	7.2
8	3.23	7.5	6.73	6.32	5.00	8.82	—	7.56	6.03

TABLE II

k VALUES FOR NITROGEN FIXATION OF CULTURES GROWN IN WARBURG FLASKS WITH VARYING OXYGEN AND NITROGEN PRESSURES

The cultures grown for 3 h.

Expt. no.	*20% oxygen Nitrogen per cent*				*10% oxygen Nitrogen per cent*			
	16	*8*	*4*	*2*	*16*	*8*	*4*	*2*
1	0.2979	0.2725	0.1927	0.1466	0.3293	0.3270	—	0.2395
2	0.3055	0.2741	0.2311	—	0.3554	0.3155	0.2956	0.2556
3	0.3316	0.3178	0.2963	0.2142	0.3923	0.3478	—	0.2963
4	0.3486	0.3140	0.2794	0.1804	0.3831	0.3524	0.3140	0.2886
5	0.3562	0.2886	0.2564	—	0.3800	0.3562	0.2886	0.2564
6	0.4061	0.3032	0.2426	0.1819	—	0.3585	0.3124	0.2257
7	0.3493	0.2679	0.2211	0.1888	0.3785	0.3170	—	0.2495
8	0.2810	0.2449	0.2242	0.1459	0.3355	—	0.2840	0.2080

conditions, analogous to the rate of any enzyme catalysed reaction. The nature of the effect of oxygen on nitrogen fixation was examined by the method of LINEWEAVER AND BURK[8] using the graphical extension described by DIXON[15].

Fig. 3 shows a plot of reciprocals of k values against reciprocals of nitrogen concentration for the data of Experiment No. 4 as a typical experiment. The lines obtained at the two oxygen concentrations have different slopes but essentially the same intercept on the vertical axis, indicating the same maximum rate of fixation with increasing nitrogen tension, that is to say a competitive inhibition by oxygen.

Statistical analysis of the whole group of data shows that of the two sets of eight lines at the two oxygen concentrations each can be represented by a single line. These two lines are shown in Fig. 4. The difference between their slopes is significant at the 1 % level, but there is no significant difference between the intercepts on the vertical axis. Thus we have a case of a classical competitive inhibition. The Michaelis constants obtained from the intercepts on the horizontal axis are 0.0107 at 10 % oxygen and 0.0229 at 20 % oxygen.

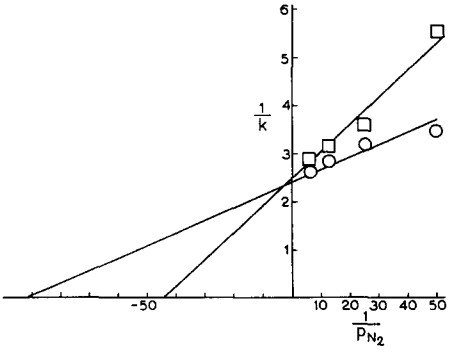

Fig. 3. LINEWEAVER-BURK plot of reciprocal of velocity constant against reciprocal of gaseous nitrogen concentration. Experiment 4. Circles: 10% oxygen. Squares: 20% oxygen.

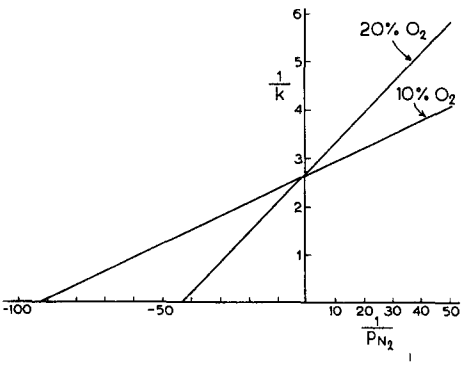

Fig. 4. LINEWEAVER plot of reciprocal of velocity constant against reciprocal of gaseous nitrogen concentration. Combined data of Experiments 1–8. Equations are: for 10% oxygen $1/k = 2.61 + 0.0279\ 1/pN_2$ for 20% oxygen $1/k = 2.68 + 0.0615\ 1/pN_2$

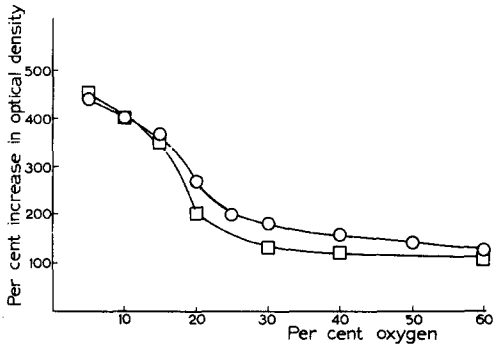

Fig. 5. Increase in O.D. of *Azotobacter* cultures grown for 4 h at different oxygen tensions. Circles, using gaseous nitrogen; squares, using ammonia as nitrogen source.

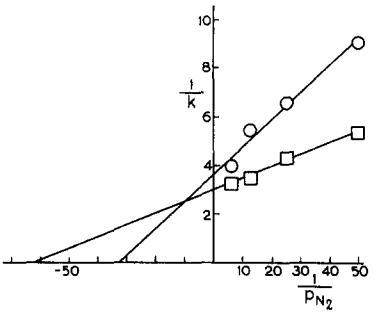

Fig. 6. LINEWEAVER-BURK plot of velocity constant against reciprocal of gaseous nitrogen concentration. Squares at 20% oxygen; circles at 30% oxygen. Culture showing almost competitive inhibition.

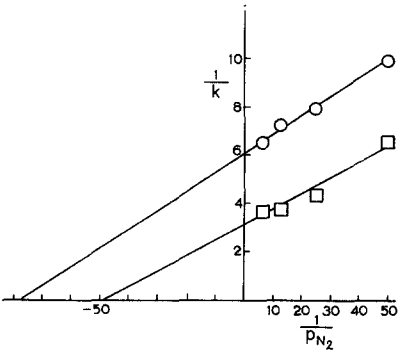

Fig. 7. LINEWEAVER-BURK plot of velocity constant against reciprocal of gaseous nitrogen concentration. Squares at 20% oxygen, circles at 30% oxygen. Culture not showing competitive inhibition.

Effects of higher oxygen concentrations

High oxygen tensions have, in addition to the specific effect on nitrogen fixation, a general effect on growth[2]. This effect is seen in Fig. 5 where increase in turbidity (O.D. at 600 mμ) is plotted against oxygen tension for cultures using gaseous nitrogen and ammonia nitrogen respectively. As stated above such data do not give an accurate measure of growth rates, but the inhibitory effect of increasing oxygen tension is clear in each case.

Because of this effect it has not been possible to obtain reliable evidence for the effect of oxygen on nitrogen fixation at 30% oxygen and above. At or about this concentration, apparently, some mechanism other than nitrogen fixation was inhibited sufficiently to become a limiting factor. The exact oxygen concentration at which this happened seemed to vary from subculture to subculture, so that some subcultures showed almost a competitive inhibition as between 20 and 30% oxygen (Fig. 6), others did not (Fig. 7).

DISCUSSION

The general inhibition of respiration and growth by high concentrations of oxygen

Azotobacter shows a decline with increasing oxygen tension both in respiration

(Fig. 1) and in growth, either on gaseous or fixed nitrogen (Fig. 5). This "normal" respiration curve for *Azotobacter*, obtained by BURK[2] and in the present experiments is difficult to interpret. BURK states that it is "unique", and it is certainly unlike the respiration curves of any of the organisms tested in this laboratory[16]. The respiratory activity of *Azotobacter*, however, is usually 5–10 times greater than that of an equivalent weight of other bacterial cells, and it is possible that the enzyme system is not saturated up to oxygen concentrations of 20–25 %.

The decline with higher oxygen pressures suggests the presence of an oxygen sensitive enzyme reaction in the respiratory chain. Such inhibitions have been described, for example, hydrogenase inactivation[17] or the pyruvate-oxidation inhibition found in *Micrococcus*[18].

The specific effect of oxygen on nitrogen fixation

The results presented show that oxygen inhibits nitrogen fixation. The nature of this inhibition has been studied by plotting the nitrogen fixation data according to the LINEWEAVER-BURK method, and it is demonstrated that a competitive relationship exists between oxygen and nitrogen during nitrogen fixation by *Azotobacter* (Fig. 4). This competitive relationship may be masked if the oxygen pressure is raised to a point where inhibition of growth of the organism limits the rate of fixation of nitrogen (Figs. 6 and 7).

When one attempts to measure an enzyme reaction rate which may itself be limited by the growth rate of the whole cell, and which is also affected by the respiratory activity of that cell, the results should be interpreted with some caution. In our experiments the response to nitrogen and to oxygen by *Azotobacter* varied significantly ($p < 0.05$) from experiment to experiment even though it was grown under rigidly controlled conditions. The Michaelis-Menten concept of enzyme reaction rates was derived from a consideration of an ideal enzyme–substrate system, and could only apply to nitrogen fixation under "whole cell" conditions provided that nitrogen fixation is the limiting rate reaction and that no restraints on the reaction are imposed by other requirements of growth. The calculation of k assumes that growth was exponential throughout the experiment, with no lag phase. Cultures were maintained in the logarithmic phase of growth during experiments, the danger of lag on dilution being reduced by a preincubation period after dilution. The overall growth rates obtained, taking into consideration the synthetic medium used, indicate that a satisfactory rate was maintained.

The value obtained for the Michaelis constant was 0.0229 at 20 % oxygen concentration. This is in agreement with that obtained by WILSON, BURRIS AND LIND[19] but not with that of WILSON AND ROBERTS[20] using $^{15}N_2$, who found a value of 6.6 % for the Michaelis constant. However, these workers were using 30 % oxygen but compared their result with a Michaelis constant of 2 % which they obtained by a respirometric method using 20 % oxygen. They concluded that the respirometric method gives erroneous results. While it is agreed that respirometric measurements may be misleading, WILSON AND ROBERTS did not consider the possibility that the difference they found might be due, in part, to the different oxygen concentrations used.

Nitrogen fixation and respiration

In an earlier publication[3] it was suggested that oxygen and nitrogen might

compete as alternative respiratory acceptors. This hypothesis was supported by BAYLISS[21] who showed that the energy changes associated with the reduction of nitrogen to ammonia are such that a substantial amount of energy could be available for work: thus, for example

$$C_6H_{12}O_6 + 4N_2(gas) + 6H_2O\ (liq) + 8H^+(aq) = 6CO_2(gas) + 8NH_4^+\ (aq);\ \Delta G' = -112\ kcal,$$

where $\Delta G'$ has been calculated on the basis of assumed biological concentrations of reactants. Thus it is possible that the microbe might gain useful energy from the nitrogen-fixation reaction. The biological reduction of nitrogen to ammonia, as BAYLISS states, requires "no 'assistance' from any other energy-providing reaction".

The suggestion that nitrogen fixation could be regarded as a form of respiration is further supported by our results, which indicate a competitive relationship between oxygen and nitrogen. HALDANE[22] considers that early forms of life were anaerobic, and that aerobic forms have developed from them. It is conceivable, too, that biological nitrogen fixation was evolved as a primitive respiratory mechanism during an anaerobic period on earth. The widespread occurrence of the nitrogen fixing ability among the anaerobic bacteria[23] lends some support to this.

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