93. MECHANISM OF BIOLOGICAL NITROGEN FIXATION

7. MOLECULAR H_2 AND THE pN_2 FUNCTION OF AZOTOBACTER¹

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A PREVIOUS report [Wyss & Wilson, 1941] demonstrated that H_2 inhibits fixation of N_2 by *Azotobacter*. Because of the apparent uniqueness of this type of inhibition, such a demonstration furnishes strong evidence that in the symbiotic and free-living N_2 fixation processes the mechanism of fixation is very similar if not identical. Further studies described in this paper provide additional support for this important conclusion.

Methods

Details of the methods used were described by Wyss & Wilson [1941]. Briefly, 25 ml. cultures of *Azotobacter* were grown under different atmospheres in litre bottles provided with a device which maintained the pO_2 constant (0.2 atm.) during the experiment. Under these conditions fixation remains logarithmic for 24–48 hr.; hence the specific rate constant of fixation, $k = d \ln(a + y)/dt$, can be readily estimated from the slope of the line obtained by plotting the log of the total N content against time. In this equation a is the initial N content of the cells and y is the increase after t hr. With cultures of *Azotobacter vinelandii*, k consistently reaches the surprisingly high values of 0.080–0.125 corresponding to a total fixation of 20–30 mg./100 ml. in 24 hr. Somewhat lower values were noted with *Az. chroococcum*. Harvests were made every 10–12 hr. often in duplicate so that each k value is based on 4–6 separate determinations. Respiration measurements were made in the Warburg apparatus by the usual technique, and micro-N determinations by the method of Johnson [1941].

The pN_2 function

A fundamental property of any enzyme system is the dependence of the rate of reaction on the concentration of substrate. Burk [1930] found that the rate of N_2 fixation by *Azotobacter* was a function of the pN_2 between 0.05 and 5 to 10 atm., being directly proportional to the pN_2 between 0.05 and 0.5 atm. Apparently these conclusions were based almost entirely on microrespiration experiments in which H_2 was used as the diluent gas. It follows then that the pN_2 function discussed by Burk [1934] is not the true one but is a complex of the effects of both N_2 and H_2 . Failure to recognize the inhibition by H_2 probably explains two characteristics of the pN_2 function which Burk thought surprising, viz. that fixation hardly occurred until a pN_2 of 0.05 atm. was present, and that

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in comparison with other enzyme-catalysed gas reactions, the Michaelis constant, K_{N_2} , was extremely high (0.21 atm.).

The pN_2 function of *Azotobacter* was determined in the presence of different 'inert' gases—argon, helium, and hydrogen. Results from the macro total N experiments are summarized in Table 1. In each experiment a 'control' was

Table 1. *Nitrogen fixation by Azotobacter as a function of the pN_2 in presence of He, A and H_2 (macro-N experiments)*

$k_{\text{air}}=100$	Relative values of k under different pN_2 in atm.								
	0.012	0.025	0.05	0.1	0.15	0.20	0.25	0.30	0.40
<i>Helium</i>									
0.095	—	—	—	93	—	96	—	103	—
0.095	—	—	—	90	—	100	—	97	—
0.064	—	70	79	93	—	96	—	—	—
0.083	70	80	82	91	—	98	—	—	—
0.071	40	—	—	76	—	—	—	—	—
0.090	52	56	—	—	—	—	95	—	—
0.125	—	—	—	85	93	—	95	—	—
0.060*	—	—	76	88	98	—	—	—	—
<i>Argon</i>									
0.037*	—	—	—	88	—	100	104	102†	—
0.043*	—	—	—	—	—	99	—	99	102
0.110	—	—	—	—	—	—	95	96†	97
0.040*	—	—	—	—	91	—	97	—	—
<i>Hydrogen</i>									
0.070	—	—	—	19	—	74	—	—	93
0.073	—	—	—	32	—	74	—	80	90
0.071	—	—	19	39	62	—	86	94†	—
0.052	—	—	—	—	—	—	66	67†	89†
0.108	—	—	—	—	—	—	82	92†	96†
0.043*	—	—	—	—	—	68	—	81	95
0.110	—	—	—	—	—	73	—	86	91
0.125	—	—	—	38	64	—	83	—	—
0.040*	—	—	—	—	46	62	74	—	—
0.060*	—	—	21	43	57	—	—	—	—

* *Az. chroococcum*; all other *Az. vinelandii*.

† 0.35 atm.

‡ 0.45 atm.

In Tables 1, 2 and 3 the figures under k_{air} are the actual specific rate constants; those opposite a given k_{air} are the relative values for the indicated atmospheres.

Table 2. *Nitrogen fixation by Azotobacter as a function of the pN_2 in absence of an inert gas (macro-N vacuum experiments)*

$k_{\text{air}}=100$	Relative k values	
	0.2	0.15
0.064	108	—
0.070	101	—
0.073	101	—
0.070	—	95
0.037*	101	—
0.081	—	92
0.052	—	92
0.083	93	—
0.108	—	95
0.110	91	—
0.040*	101	—
0.125	94	—
0.060*	—	90†
0.072	—	94
0.101	—	93

* *Az. chroococcum*; all others *Az. vinelandii*.

† 0.1 atm.

included at some value of the pN_2 , usually, 0.2 atm., in which no inert gas was added but instead the cultures were grown under a partial vacuum; the pO_2 for such cultures was kept constant at 0.2 atm. by supplying pure O_2 through a column of mercury. These values are given in Table 2.

The averages of the data in these two tables, together with values from a few miscellaneous experiments, are shown in Fig. 1, which emphasizes the striking

Fig. 1. The pN_2 function of *Azotobacter* based on macro total N experiments. This figure is compiled from over 100 separate estimations of the rate of fixation in different gas mixtures. The individual points shown are the means of 3-7 estimations; each of these is based on analyses of 4-6 cultures.

difference in the effect of the pN_2 in the presence and absence of H_2 . If He, A, or a vacuum replaces the abstracted N_2 , no consistent change in the rate of fixation is observed until the pN_2 is lowered to approximately 0.15 atm.; the rate then decreases slowly with the pN_2 so that at 0.01 atm. the value of k is still is about 50 % of the maximum. In the presence of H_2 , however, a significant reduction in the rate of fixation is noted at a pN_2 of 0.45 atm. ($pH_2=0.35$ atm.). As more H_2 is added, the rate drops so rapidly that at a pN_2 of 0.05 atm. ($pH_2=0.75$ atm.) fixation is barely detectable in 48 hr. That this effect of H_2 is specifically associated with the N_2 fixation reaction is indicated by the data in Table 3 which show that the uptake of NH_4NO_3 -N is independent of H_2 in the atmosphere.

Table 3. Assimilation of NH_4NO_3 by *Azotobacter* as a function of the pN_2 in the presence of H_2 (macro-N experiments)

$k_{air}=100$	Relative values of k for different pN_2 in atm.				
	0.025	0.05	0.10	0.20	0.30
0.115	—	103	101	99	102
0.056	—	102	104	111	97
0.109	99	100	97	98	—
0.093	—	99	94	98	—
0.031*	—	81	93	97	—

* *Az. chroococcum*; all others *Az. vinelandii*.

The values obtained with H_2 in these macro-N experiments which lasted 24-48 hr. are in reasonable agreement with those reported by Burk who estimated uptake of free or combined N by microrespiration measurements over

periods of 4–8 hr. It was believed desirable, however, to check at least the critical values indicated by the curves in Fig. 1 by the microrespiration technique. Results from typical experiments are summarized in Table 4. In these experiments duplicate or triplicate flasks were taken of each treatment, and the best

Table 4. Nitrogen fixation by *Azotobacter* as a function of the pN_2 in the presence of *A*, *He*, and *H_2* (microrespiration experiments)

Diluent gas	Treatment				Air control
	k values for different pN_2				
	0.05 atm.	0.10 atm.		0.15 atm.	
H ₂		0.133 0.132	0.133		
He	0.219 0.219	0.229 0.252	0.240	0.263 0.248 0.232	0.306
	0.3 atm.	0.4 atm.	0.5 atm.	0.6 atm.	
H ₂	0.208 0.211	0.245	0.260 0.243	0.261 0.265	0.263
He	0.278 0.303				0.277 0.287
	0.4 atm.	0.5 atm.	0.6 atm.		
H ₂ *	0.359 0.364 0.353	0.350 0.367 0.368	0.397 0.397 0.389	0.440 0.442 0.422	0.431
	0.01 atm.	0.025 atm.	0.05 atm.		
He	0.104 0.103 0.105	0.143 0.144	0.163 0.163	0.290 0.293 0.277	0.287
	0.01 atm.	0.025 atm.	0.05 atm.		
He	0.136 0.137 0.138	0.155 0.161 0.155	0.193 0.185	0.275 0.286 0.270	0.277
		0.05 atm.	0.10 atm.		
H ₂ (NH ₄ NO ₃)		0.278 0.254	0.266	0.260 0.263 0.264	0.290 0.262 0.272

* *Az. chroococcum*; all others *Az. vinelandii*.

straight line for each flask was determined by the usual statistical procedure. In a given experiment the variances, s^2 , for the slopes of the different lines were shown to be homogeneous; therefore these could be pooled and a common variance for the experiment calculated. When this was done, it was readily demonstrable that growing cultures in an atmosphere with a pH_2 of 0.2 atm. or more decreased significantly the mean value of k in comparison with the air control. It appears then that when the $pN_2:pH_2$ ratio is high, the microrespiration technique is more sensitive for detection of inhibition than is the macro-N method. The final experiment cited in Table 4 demonstrates that the effect of H_2 is specifically concerned with the fixation system since the values of k for uptake of NH_4NO_3 -N (estimated by the increase in rate of respiration of *Azotobacter* with time) are independent of the pH_2 . As was observed in the macro-N experiments whenever helium was used as the diluent, the rate of fixation was significantly lowered only after the pN_2 was reduced to about 0.15 atm. Even at a pN_2 of approximately 0.01 atm. the rate of fixation reached

40–50% of the value in air. As is evident in Fig. 2, the pN_2 curves based on the microrespiration data are very similar in the presence and absence of H_2 to the corresponding functions derived from the macro-N experiments.

Fig. 2. The pN_2 function of *Azotobacter* based on microrespiration experiments. Each point represents the mean of 2–6 determinations in separate experiments; in each experiment duplicate or triplicate flasks were taken.

In some of the respiration experiments several flasks of each treatment were taken, and periodically one was removed for micro-N determinations. The values of k together with the necessary statistical constants for estimation of the significance of observed differences are given in Table 5. The micro-N determinations are less accurate than the respiration data, but the two sets of results

Table 5. Nitrogen fixation by *Azotobacter* as a function of the pN_2 (micro-N experiments)

Composition of gas mixture*			Respiration			Micro-N		
pN_2	pHe	pH_2	k value	N	S_k †	k value	N	S_k †
atm.	atm.	atm.						
0.8	—	—	0.273	18	0.0138	0.148	4	0.0131
0.15	0.65	—	0.233	18	0.0115	0.149	4	0.0051
0.15	—	0.65	0.139	18	0.0095	0.080	4	0.0150
0.8	—	—	0.226	18	0.0104	0.188	6	0.0099
0.3	0.5	—	0.210	27	0.0087	0.193	8	0.0163
0.3	—	0.5	0.171	19	0.0085	0.117	8	0.0112
0.8	—	—	0.232	18	0.0087	0.187	5	0.0104
0.2	0.6	—	—	—	—	0.206	5	0.0267
0.1	0.7	—	0.205	18	0.092	0.168	4	0.0147

* $pO_2 = 0.2$ atm. in all mixtures.

† $S_k = S_k^2 / \Sigma (x - \bar{x})^2$. To test difference between two values of k , calculate $S' = \sqrt{(S_{k_1}^2 + S_{k_2}^2)}$.

Then $t = \frac{k_1 - k_2}{S'}$ with $N_1 + N_2 - 4$ degrees of freedom.

parallel one another sufficiently to conclude that increase in O_2 uptake probably serves as a reliable index for the estimation of N_2 fixation. In general the k values based on respiration are consistently higher than those based on total N, but the relative values do not differ materially so that the conclusions drawn from either type of data would be the same. Specifically, they show that reduction

of the pN_2 to 0.1–0.2 atm. causes only a small decrease in the rate of N_2 fixation by *Azotobacter* in the presence of helium but a marked decrease in the presence of H_2 .

Nature of the inhibition

The type of inhibition by H_2 —whether competitive or non-competitive—was determined by comparing the rates of N_2 fixation at different values of the pN_2 and pH_2 . As discussed in detail by Wilson *et al.* [1941], if the concentration of substrate (pN_2) is kept constant the relative rate of fixation in the presence of inhibitor (k/k_i) is a linear function of the concentration of inhibitor (pH_2) with an intercept of unity. If the inhibition is competitive, the slopes of the lines obtained when k/k_i is plotted against the pH_2 for different values of the pN_2 are significantly different. The method is illustrated in Fig. 3 in which the data from Exps. II and III of Table 6 are used.

Fig. 3. Competition of H_2 with N_2 in *Azotobacter* N fixation experiments. The significant difference in the slopes of the lines obtained when k/k_i is plotted against the pH_2 for different values of the pN_2 indicates that the inhibition is competitive.

Table 6. *Nature of inhibition by H_2 (macro-N experiments)*

Exp.	pN_2 atm.	Slope of line	t value for difference in slopes
I	0.2	0.4170	2.97
	0.4	0.2235	
II	0.15	0.9118	5.60
	0.30	0.4383	
III	0.15	1.0709	5.27
	0.30	0.4959	

Since each line contributes 2 degrees of freedom, t must be at least 2.78 for a significant difference (19:1 odds). As this value was exceeded in all the experiments, it is concluded that the slopes of the lines differed significantly; therefore the inhibition is competitive.

Reversibility of inhibition

The reversibility of the inhibition by H_2 as well as its complete removal by combined N was demonstrated by a macro-N experiment with *Az. vinelandii* in which the conditions under which assimilation of N_2 occurred were altered

during the experiment. The treatments and results are shown in Fig. 4. The values of k were: air throughout, 0.080 (line 1); H_2 throughout, 0.037 (line 6). When cultures originally under air were shifted at the end of 32 hr. to the atmosphere containing H_2 (line 3), the rate of fixation decreased to that of H_2 throughout. Likewise, when after 32 hr. cultures in the H_2 atmosphere were transferred to air (line 5), the rate increased to that of cultures kept in air

Fig. 4. Reversibility of H_2 inhibition of N fixation by *Azotobacter*. (H_2 indicates: pH_2 , 0.65 atm.; pO_2 , 0.20 atm.; pN_2 , 0.15 atm.)

1. Air throughout.
2. 10 mg. fixed N under H_2 .
3. Air to 32 hr., change to H_2 .
4. H_2 to 32 hr., 20 mg. N added, continued under H_2 .
5. H_2 to 32 hr., change to air.
6. H_2 throughout.

throughout the experiment. Moreover, if excess NH_4NO_3 -N was added to cultures kept under the H_2 atmosphere, the inhibition by H_2 was removed even though incubation continued under the same atmosphere (line 4). Finally, if a small quantity of combined nitrogen was added initially to the cultures, H_2 did not inhibit until all fixed N was utilized (line 2).

Comparison of effects of H_2 on fixation by red clover and *Azotobacter*

Additional evidence that H_2 affects the symbiotic and asymbiotic N fixation systems in a similar manner was obtained by an experiment in which red clover plants and *Azotobacter* cultures were kept in the same environment. The plants were grown in 9 l. pyrex bottles under the gas mixture: pO_2 , 0.2 atm.; pN_2 , 0.3 atm. In one series (*Vacuum*) no other gas was supplied, in a second (*Hydrogen*) H_2 was added to 1 atm. The substrate was a N-poor sand plus N-free Crone's solution. In each series one-half of the plant cultures were inoculated with *Rhizobium trifolii* 209; the remainder were supplied with NH_4NO_3 . After 10 weeks 200 ml. tubes containing 50 ml. of Burk's medium inoculated with *Azotobacter vinelandii* were placed in representative bottles of each series. NH_4NO_3 was added to the medium whenever the tube was placed in a red clover culture furnished with this source of N. The *Azotobacter* tubes were wrapped in light-tight paper so that the bottles could be kept in a greenhouse. The bottles were then stoppered, and the experimental atmospheres were replaced. The *Azotobacter* cultures metabolized the sucrose in the medium to produce CO_2 for the plants; the plants assimilated this with production of O_2 thus keeping the pO_2 reasonably constant. A type of 'symbiosis' resulted which maintained the N-fixing systems of the *Azotobacter* and red clover plants in the same gaseous environment.

After one week the bottles containing both red clover and *Azotobacter* cultures were harvested, and the experiment was repeated the following week with the remaining bottles. During this second week, the temperature of the greenhouse was raised from 24° to 28°, and helium was added to 1 atm. in the bottles of the *Vacuum* series. The following data summarize the results; the values given (mg. total N per culture) are the means of triplicate samples:

Treatment	<i>Azotobacter</i>		Red clover	
	N ₂	NH ₄ NO ₃	N ₂	NH ₄ NO ₃
I. First week:				
<i>Vacuum</i>	6.88	5.10	54.7	70.0
<i>Hydrogen</i>	3.70	5.60	34.5	62.8
II. Second week:				
<i>Helium</i>	13.10	9.42	68.9	102.2
<i>Hydrogen</i>	9.90	9.35	44.1	103.1

Although the conditions were not ideal for fixation of N₂ by *Azotobacter* (low temperature, small surface exposed), the type of result was identical with that observed in the experiments made under more optimum conditions. The data of this combination trial confirm the conclusion based on the extensive observations made with the separate N-fixing systems: fixation of N₂ by both inoculated red clover plants and *Azotobacter* is inhibited by H₂ but assimilation of NH₄NO₃-N is not affected.

DISCUSSION

The results presented in this paper together with those previously reported provide strong support for the view that the enzyme systems responsible for the fixation reaction in inoculated red clover plants and in the free-living *Azotobacter* are identical. Before discussing the significance of this conclusion, we shall briefly analyse the major evidence in its favour.

The pN₂ function. The manner in which the rate of reaction changes with concentration of substrate is very similar for both systems. If the curves in Figs. 1 and 2 are compared with that for the symbiotic system [Wilson, 1940], a close resemblance is noted. In both cases the rate of reaction is independent (95–100 % of maximum) of the pN₂ until the latter is lowered to about 0.15 atm., and the apparent Michaelis constants (K_{N_2}) are of the same order of magnitude. Estimation of the K_{N_2} for *Azotobacter* from the results of the macro total N experiments by the method of Lineweaver & Burk [1934] gave a value of 0.012 atm.; the less extensive microrespirometer data indicated a K_{N_2} of 0.015 atm. Great precision, however, is not claimed for these values since the number of experiments on which the estimates are based is not large, but they are sufficiently accurate for our purposes. In our previous studies with red clover a K_{N_2} of approximately 0.05 atm. was obtained for the symbiotic system. This figure is very likely a maximum value since the technical conditions which must be used in the plant studies may cause the rate of diffusion of the gases into the nodule to affect the determination of the K_{N_2} . Diffusion may likewise influence the estimates of the K_{N_2} for the *Azotobacter* system, but in view of the close agreement between the values obtained with quiescent macrocultures and the vigorously shaken microcultures, this appears less likely. It seems probable that the values of the K_{N_2} for both systems are fairly low (ca. 0.01–0.02 atm.), are of the same order of magnitude and do not differ greatly from those of other enzyme-catalysed gas reactions.

Inhibition by H₂. Not only does H₂ inhibit both systems, which in itself is rather unusual, but the quantitative aspects of the inhibition are similar. If

the rate of N_2 fixation by red clover plants is plotted against the pH_2 in the atmosphere, a straight line results [Wilson *et al.* 1938], whereas the corresponding curve for the *Azotobacter* system appears to be hyperbolic (Figs. 1 and 2). This difference in the type of curve is reflected in the K_{H_2} values of the two systems. On the assumption that the K_{N_2} for *Azotobacter* is 0.01 atm., the data from the three experiments of Table 6 give a mean value of 0.08 atm. for the K_{H_2} . The K_{H_2} for the symbiotic system in red clover plants is 0.14 atm. on the basis of a K_{N_2} of 0.05 atm. [Wilson, 1940]. That is, the ratio of the affinity of the N_2 -fixing system for N_2 to that for H_2 is 8 in *Azotobacter* and 3 in red clover. In the symbiotic system, however, the values of the Michaelis constants may be affected to some extent by the relative rates of diffusion of the gases into the nodules so that this comparatively small (2 to 3-fold) difference may not be significant. In addition, the fact that the inhibition is competitive and is readily reversible in both instances affords strong support for the view that the two systems respond to H_2 in an identical manner.

The identification of the symbiotic and asymbiotic N_2 -fixing enzyme systems through comparison of their physical-chemical characteristics has important implications for the general field of biological mechanisms as well as for the specific problem of the mechanism of biological N fixation. This application of the physical-chemical approach to a problem of biological mechanism demonstrates its usefulness for investigation of enzyme systems which are not readily separated from the living cell. It should be noted that, *a priori*, one could not conclude that the responsible enzyme systems in these two biological agents are similar since obvious differences exist in the symbiotic and asymbiotic methods for N_2 fixation—differences which may or may not reflect fundamental dissimilarities in the mechanism of the reaction. The question naturally arises: do other biological agents which fix N_2 —*Clostridia*, for example—possess a mechanism similar to that of *Azotobacter* and inoculated red clover plants? Investigations similar to those described in this and previous reports should provide the answer. Since the *Clostridia* liberate H_2 during breakdown of carbohydrates, it will be of interest to learn whether this cellular reaction affects the concurrent one of N_2 fixation.

SUMMARY

Based on macro total nitrogen rate experiments, the pN_2 function of *Azotobacter* is characterized by the complete independence of the rate of fixation of the pN_2 when the latter exceeds 0.2 atm. Below this value the rate of fixation decreases slowly; a K_{N_2} of approximately 0.01 atm. is indicated. In the presence of H_2 , however, a decrease in the rate of fixation is observed when the pN_2 is reduced to 0.45–0.5 atm. The decrease in the rate with pN_2 (increasing pH_2) proceeds very rapidly so that at a pN_2 of 0.05 atm. fixation is barely detectable. This effect of H_2 is not observed with cultures supplied with NH_4NO_3 ; hence the effect is specific for the nitrogen fixation process.

The pN_2 functions of *Azotobacter* in the presence of different diluent gases determined by microrespiration experiments are practically identical with those determined by the macro total nitrogen experiments.

The inhibitory action of H_2 is reversible and competitive. The K_{H_2} value is approximately 0.08 atm., i.e. the affinity of the N_2 -fixing system for N_2 is 8 times that for H_2 .

These physical-chemical properties of the N_2 -fixing system in *Azotobacter* are very similar to those we have previously established for red clover plants

inoculated with the specific root nodule bacteria. It is therefore concluded that the responsible enzyme systems in these diverse biological agents—free-living *Azotobacter* and symbiotic plant plus bacteria—are identical.

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