MOLECULAR H_2 AND THE PN_2 FUNCTION OF AZOTOBACTER*

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In 1941, Wyss *et al.*[1] published a paper under the foregoing title in which evidence was presented that the specific inhibition by molecular H_2 of nitrogen fixation by *Azotobacter vinelandii* was competitive, as had been previously established for the symbiotic system in red clover.[2] These researches provided the first example of the recently described diversity of the nitrogenase system for reaction with various substrates such as nitrous oxide, acetylene, azide, cyanide, and isocyanide.[3-8] Using the microrespirometric technique, Parker and Dilworth[9] re-examined the type of inhibition by H_2 and concluded that it was not competitive. To explain the difference in results, they suggested that H_2 induced a lag in cultures grown at a low pN_2 but not at a high pN_2, causing deviation from exponential growth, i.e., nitrogen fixation, and that uptake of O_2 is not consistently correlated with the nitrogen content of the cell. Early investigators[10, 11] using this technique were quite aware of these limitations and in their papers emphasized the necessity of calculation of the rate constants only for the period of a demonstrated exponential growth and for periodic verification of the technique by estimation of total N.

Moreover, these suggested explanations were not relevant, since our published data were from macro-experiments in which rates of fixation were measured during the exponential phase of growth by estimation of total nitrogen. This technique, however, was open to another criticism:[9] that inadequate control of the O_2 supply either in microrespiration experiments or in macrocultures might affect the results since O_2 is a competitive inhibitor of nitrogen fixation by the azotobacter.[12] The development by Bulen *et al.*[13] of a cell-free system from *Azotobacter vinelandii* capable of N_2-fixation provided an opportunity to re-examine this question under conditions that are not complicated by the presence of O_2.

Materials and Methods.—One-liter cultures of *Azotobacter vinelandii* OP contained in 2-liter Erlenmeyer flasks were grown on a modified Burk's N-free medium of the following composition: KH_2PO_4, 0.2 gm; K_2HPO_4, 0.8 gm; $MgSO_4 \cdot 7H_2O$, 0.2 gm; $CaCl_2 \cdot 2H_2O$, 0.09 gm; sucrose, 20 gm; 1 ml of an Fe-Mo solution (1 mg Fe and 0.1 mg Mo/ml); H_2O, 1000 ml. After 16–18 hours of incubation at 30° on a rotary shaker, the cells were harvested by centrifugation at 0–4° in an International refrigerated centrifuge at 2000 rpm for 30 minutes. The cells were washed once or twice with 30–40 ml cold 0.025 *M* phosphate buffer, pH 7.0, centrifuged at 8000 $\times$ *g* for five minutes, and the pellet was weighed. They were resuspended in the same buffer at a ratio of 3 ml buffer per gram cell paste. The cells in this suspension were broken in a precooled French pressure cell, then centrifuged at 10,000 $\times$ *g* for ten minutes. The resulting supernatant fraction containing from 25–35 mg protein/ml had a pH of about 6.8 and was free from whole cells.

The specific activities (nanomoles NH_3 formed/mg protein/min) were estimated by the method of Bulen *et al.*[13] with minor modifications. The assays were made in 20-ml serum bottles shaken on a reciprocal shaker (approximately 200 oscillations/min) for 25 minutes at 30°. The nitrogen fixed (corrected for that formed

TABLE 1

THE pN_2 FUNCTION OF NITROGEN FIXATION BY CELL-FREE EXTRACTS OF *Azotobacter vinelandii*

pN_2 (atm)	N fixed* (μg)	Specific activity
0.05	4.75, 5.24	2.06, 2.27
0.075	7.62, 7.83	3.30, 3.39
0.10	9.01, 8.24	3.90, 3.57
0.15	11.47, 11.05	4.97, 4.78
0.30	15.31, 13.21	6.63, 7.18
0.50	17.48, 17.06	7.57, 7.39

* Corrected for helium control, 2.17 μg.

in a helium control) was estimated by the technique described by Dilworth *et al.*;[14] the validity of this method of assay was established by use of $^{15}N_2$ as a tracer. Details of the experimental procedures and results are in the thesis of Strandberg.[15]

The p_{N_2} Function.—Experiments designed to estimate the K_{N_2} for the cell-free system led to unexpected high values in comparison with those previously observed with intact cells. The detailed data in Table 1 provide information on the type of agreement between duplicate samples; a double reciprocal plot of these data (Fig. 1) resulted in an estimate for the K_{N_2} of 0.16 atm. Combined data from five experiments are shown in Figure 2; the estimate of K_{N_2} = intercept/slope for the line of best fit is 0.157 atm, a value in excellent agreement with that recently published by Hardy and Knight,[7] who used a heated extract of *A. vinelandii* (specific activity, 10.8). For intact cells the estimate was 0.01–0.02 atm.[16]

Although many of the estimated K_{N_2} values for the cell-free N-fixing systems have been consistently higher than those obtained with intact cells, the disparity with other organisms has not been so great as observed in our experiments with *A. vinelandii* (Table 2). Examination of the data in this table reveals that often the apparent K_{N_2} varies with the experimental conditions used for its estimation, including the method for determining k, the specific rate constant of fixation. For growing cells, it is essential that the estimate of k be based on the period of exponential nitrogen fixation. Calculation of k from the formula $k = 1/t$ [log (N at time t/initial N)] can lead to serious error unless the experimental controls ensure that the time interval of the observations is during this period. With the azotobacter

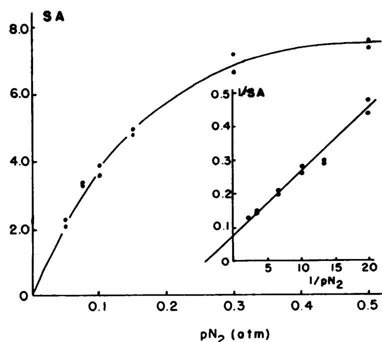

FIG. 1.—The pN_2 function of nitrogen fixation by cell-free extracts of *Azotobacter vinelandii* OP. Data from Table 1. K_{N_2} = 0.16 atm.

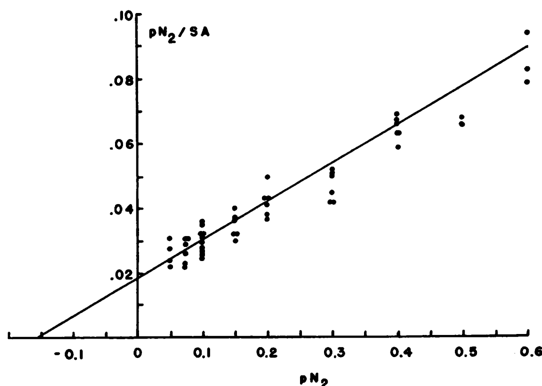

FIG. 2.—Estimation of the K_{N_2} for nitrogen fixation by cell-free extracts of *A. vinelandii* OP. Data from five separate experiments. K_{N_2} = 0.16 atm.

TABLE 2

COMPARISON OF K_{N_2} VALUES FOR N_2-FIXATION BY INTACT CELLS AND CELL-FREE EXTRACTS

	Intact Cells			Cell-Free Extracts		
Organism	K_{N_2}	Expt. conditions*	Reference	K_{N_2}	Expt. conditions*	Reference
A. vinelandii	0.01–0.02	(*a*, *b*)	1	0.16	(*i*)	This paper
	0.02	(*a*)	17	0.16	(*i*)	7
	0.066	(*c*)				
	0.01	(*d*) (10% O_2)	9			
	0.023	(20% O_2)				
C. pasteurianum	0.03	(*e*)	18	0.037	(*j*)	14
	0.076	(*f*)	19	0.08	(*k*)	19
				0.165	(*j*)	20
B. polymyxa	0.03	(*e*)	23	0.15	(*i*)	21
K. pneumoniae	0.095	(*g*)	24	0.12	(*i*)	22
Soybean nodules	0.025	(*h*)	25			
Soybean bacteroids				0.056	(*i*)	26

* *Key:* (*a*) Microrespirometric; *k* from slope of line.
(*b*) Still macrocultures, total N; *k* from slope of line.
(*c*) Uptake of $^{15}N_2$ by macrocultures; *k* by formula.
(*d*) Shaken microcultures; total N; *k* by formula (verified culture in exponential phase by O_2 uptake measurements).
(*e*) Shaken macrocultures; total N; *k* by formula.
(*f*) Shaken microcultures; *k* based on uptake of $^{15}N_2$ in short-time trials (45 min).
(*g*) Shaken macrocultures; total N; *k* by formula (verified culture in exponential phase by turbidity measurement; K_{N_2} estimated from Fig. 4 in reference).
(*h*) Uptake of $^{15}N_2$ by nodule slices in microrespiration flasks; *k* by formula.
(*i*) Technique of Bulen *et al.*;[13] dithionite as electron donor, creatine phosphate as source of high-energy phosphate.
(*j*) Technique of Mortenson;[20] H_2 as electron donor, acetyl phosphate as source of high-energy phosphate.
(*k*) Technique of Carnahan *et al.*;[27] pyruvate as source of both electrons and high-energy phosphate.

the most convenient technique is the microrespirometric method introduced by Burk[10] since k is readily determined by the slope of the line when log uptake of O_2 is plotted against time. However, as has been noted, oxygen uptake does not always consistently measure the N content of the cells.

Using the microrespirometric technique, Wilson and Roberts[17] obtained a K_{N_2} for *Azotobacter vinelandii* of 0.015 atm, but when the estimation was based on uptake of $^{15}N_2$, the value rose to 0.066 atm. The usual explanation for such a discrepancy based on the indirect nature of the respirometric technique led them to regard the $^{15}N_2$ data as more reliable and to describe the data presented as the first accurate direct determination of the Michaelis constant from nitrogen uptake measurements. Our own experiments[1, 16] included estimates based on both microrespirometric data and total nitrogen determinations of macrocultures, estimates that were in close agreement. Parker and Scutt[12] reported a K_{N_2} of 0.01 atm for *Azotobacter vinelandii* when a pO_2 of 0.1 atm was used; the value rose to 0.023 when the pO_2 was increased to 0.2 atm. Since a pO_2 of 0.3 atm was used in the isotopic experiments and 0.2 atm in the microrespirometric,[17] they suggested that this difference may have been a factor.

The values for the K_{N_2} for nitrogen fixation by cell-free extracts of *Clostridium pasteurianum* W-5 have varied fourfold from 0.037 to 0.165 (see *Note added in proof*), but as is noted in the table, different conditions were used by the investigators.[14, 19, 20] Two estimates are available for intact cells, 0.03 and 0.08 atm.[18, 19] A preliminary examination of the cell-free system of *Bacillus polymyxa*[21] and for *Klebsiella pneumoniae*[22] in our laboratory has indicated values similar to those observed for *A. vinelandii* as compared with estimates of 0.03 and 0.095, respectively, for the intact cells.[23, 24]

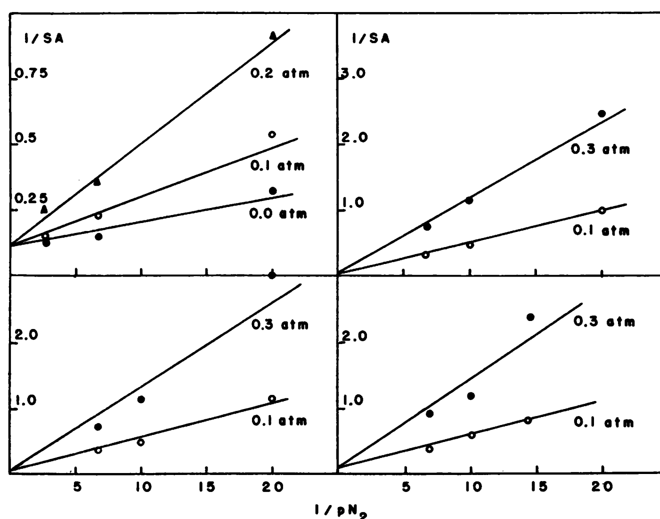

FIG. 3.—Hydrogen inhibition of nitrogen fixation by cell-free extracts of *A. vinelandii* OP. The Lineweaver-Burk plot in the upper left-hand corner is based on the data of Table 3.

Somewhat better agreement has been obtained for the symbiotic system of the soybean plant. Burris *et al.*[25] reported 0.025 atm as the K_{N_2} for soybean nodule slices, and recently Koch *et al.*[26] found a value of 0.056 atm for the cell-free system prepared from the bacteroids isolated from the nodules. Interestingly, the first estimation[2] of a K_{N_2} for a symbiotic system made on red clover plants in growth experiments lasting several weeks led to a value of 0.05 atm.

Type of Inhibition by H_2.—Results of experiments to determine the type of inhibition by H_2 are given in Figure 3 and Table 3. The double reciprocal Lineweaver-Burk plots of the data obviously suggest competitive inhibition. The fit of the points to straight lines with a common intercept is reasonably good except for some of those of the higher pH_2/pN_2 ratios. However, these points are the least reliable, partly because small errors in the assumed composition of the gas mixtures lead to relatively large errors in the abscissa value of the reciprocal plot. More important, however, the estimation of the specific activity is least accurate for these high ratios, since the quantity of nitrogen fixed is about the same as the background value of the control.

TABLE 3

HYDROGEN INHIBITION OF NITROGEN FIXATION BY EXTRACTS OF *Azotobacter vinelandii* OP

pN_2	pH_2	N fixed (μg)	Specific activity
0.1	0.0	6.74, 6.69	3.16, 3.13
0.3		12.7, 15.0	5.94, 7.03
0.6		16.3, 15.4	7.65, 7.20
0.1	0.1	4.67, 4.67	1.71, 1.71
0.3		13.06, 10.40	4.78, 3.81
0.6		19.33, 19.20	6.23, 6.08
0.1	0.2	2.55, 2.07	1.19, 0.97
0.3		5.92, 6.20	2.77, 2.90
0.6		8.27, 8.27	3.87, 3.87

Helium controls, 2.21, 2.60.

The conclusion that the inhibition is competitive, as was recorded in our initial paper with whole cells,[1] appears to be justified; furthermore, it is consistent with the recent findings of other workers for the clostridial[14, 28] and soybean[26] systems. Nevertheless, considerations of certain limitations of the experimental procedure suggest that final decision should be reserved until these are resolved. The differences in the K_{N_2} values obtained with intact cells in comparison with cell-free extracts are unexplained. One can postulate that N_2 is more soluble in the cellular materials than in the buffer solution of the extract; or possibly the intact cell has a specific organized transport system for concentration of the substrate at the site of the enzyme. Without experimental support such speculations may distract from other explanations that may arise from deficiencies in the technique.

One obvious and readily achieved improvement will be examination of more highly purified enzyme preparations with considerably higher specific activities. However, a second complicating factor may not be easily overcome—the role of H_2 not only in the inhibition experiments but also in those dealing with the pN_2 function. The experiments with cell-free extracts of *C. pasteurianum* have had this gas present either as a reductant or as a product from pyruvate. Although an artificial electron donor was used in the experiments with the other organisms, including azotobacter, H_2 is still present in the same sense as in the clostridial pyruvate system because of the activity of the ATP-dependent H_2-evolving system in the extracts.[13] Fixation at low values of the pN_2 (less than 0.1 atm) is often low and erratic; a possible explanation is that with low levels of N_2 more electrons are diverted to the production of H_2 and fixation is inhibited to a greater extent. Obviously, the quantity of H_2 formed is so small that it does not influence the composition of the gas mixture provided, but the liberation of H_2 at or near the site of fixation might have an appreciable effect. It is recalled that the first estimate of the K_{N_2} by Burk *et al.*[29] led to a value of 0.21 atm, but this value was shown to be in error because of the inhibition by H_2 gas used as a diluent.[1] Although the similarity of this estimate with the high values found for the cell-free extracts may be fortuitous, the possible complication of the enzyme systems concerned with H_2 metabolism in the nitrogen-fixing extracts should not be entirely dismissed. An example of how the role of H_2 can vary with the experimental conditions used is provided by Lockshin and Burris:[28] inhibition of the clostridial system by H_2 was competitive at optimal phosphate concentrations but uncompetitive at higher phosphate concentrations.

Note added in proof: Figures 3, 4, and 6 in the paper by Lockshin and Burris[28] provide three additional estimates to be made of the K_{N_2} for cell-free preparations of *C. pasteurianum*: 0.08, 0.16, and 0.2 atm. T. O. Munson (private communication) of the Burris group has recently estimated the K_{N_2} of cell-free preparations of *Rhodospirillum rubrum* to be 0.07 atm.

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