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A Nitrogen Pressure of 50 Atmospheres Does Not Prevent Evolution of Hydrogen by Nitrogenase

Abstract. *The effect of a partial pressure of nitrogen of 50 atmospheres (5065 kilopascals) on the hydrogen evolution reaction of nitrogenase has been investigated. Evolution of hydrogen was not blocked completely by 50 atmospheres of nitrogen in any of four experiments; rather, 27.3 ± 2.4 percent of the total electron flux through nitrogenase was directed toward production of hydrogen. The ratio of hydrogen evolved to nitrogen fixed was close to 1:1, which implies that hydrogen evolution is obligatory in the fixation of molecular nitrogen by nitrogenase.*

Nitrogenase catalyzes the reduction of N_2 to NH_3 and consists of two electron-transferring proteins, dinitrogenase reductase (iron protein) and dinitrogenase (molybdenum-iron protein). Nitrogenase is found in strictly aerobic, microaerophilic, facultative, and strictly anaerobic prokaryotes, and, depending upon the organism and the growth conditions, these organisms may acquire energy heterotrophically or photoautotrophically (*1*). Dinitrogenase reductase transfers a single electron at a time to dinitrogenase (*2*) with the concomitant hydrolysis of two molecules of adenosine triphosphate (ATP) to yield two molecules of adenosine diphosphate plus orthophosphate (*1*, *3*). Dinitrogenase, with its multiple iron-sulfur centers and its FeMoco prosthetic group or groups (*4*, *5*), both stores electrons and transfers reducing equivalents to substrates (*4*).

Nitrogenase evolves H_2 and reduces N_2 to NH_3 (*6*, *7*). Uptake hydrogenases present in many nitrogen-fixing bacteria can recycle H_2 evolved by nitrogenase and recover part of its energy as ATP or reducing equivalents, or both (*7*). In experiments with cell-free nitrogenase from *Azotobacter vinelandii*, Rivera-Ortiz and Burris (*8*) observed a Michaelis constant (K_m) of 0.136 atm for N_2 and showed that increasing the partial pressure of N_2 (pN_2) between zero and 1 atm decreased H_2 evolution significantly. Extrapolation of their data for moles of H_2 evolved versus pN_2 to infinite pN_2 indicated that most of the electron flux through the enzyme was directed toward NH_3 formation but that H_2 evolution still accounted for 13 to 23 percent of the electron flow. These results implied that infinite pN_2 was unable to block evolution of H_2 completely. In addition, their observation that the percentage of the electron flux allocated to H_2 evolution was near the theoretical 25 percent predicted by a stoichiometry of one molecule of H_2 evolved per one molecule of N_2 fixed suggested that the ratio of H_2 evolved to N_2 fixed might be 1:1 at infinite pN_2. Recently mechanisms for nitrogenase catalysis have been proposed that assume one molecule of H_2 is evolved per molecule of N_2 fixed (*9*). However, as far as we know, no one has tested the prediction of Rivera-Ortiz and Burris (*8*) experimentally to determine whether their extrapolation is justified or whether evolution of H_2 is prevented by a very high pN_2. In this report we present the results of experiments that test the effect of 50-atm pN_2 on the H_2 evolution reaction of nitrogenase.

Azotobacter vinelandii OP was grown in a 300-liter fermentor on the nitrogen-free medium described by Strandberg and Wilson (*10*). The nitrogenase proteins were purified by the method of Hageman and Burris (*11*) to specific activities of 2100 and 1864 ± 181 nmole of acetylene reduced per minute per milligram of protein for dinitrogenase reductase and dinitrogenase, respectively. Protein was determined by the method of Goa (*12*) with bovine serum albumin used as a standard. Nitrogenase concentrations were selected to avoid any dilution effect (*13*), and reactions were conducted with a 20-fold molar excess of dinitrogenase reductase over dinitrogenase. These precautions ensured maximal electron flow through dinitrogenase during the reactions. High electron flow rates favor reduction of N_2 and decrease formation of H_2 (*11*). Product formation was linear for the period of the assays.

The high-pressure reaction vessel (Fig. 1) consisted of a 22-ml reaction vessel made of 316 stainless steel with a

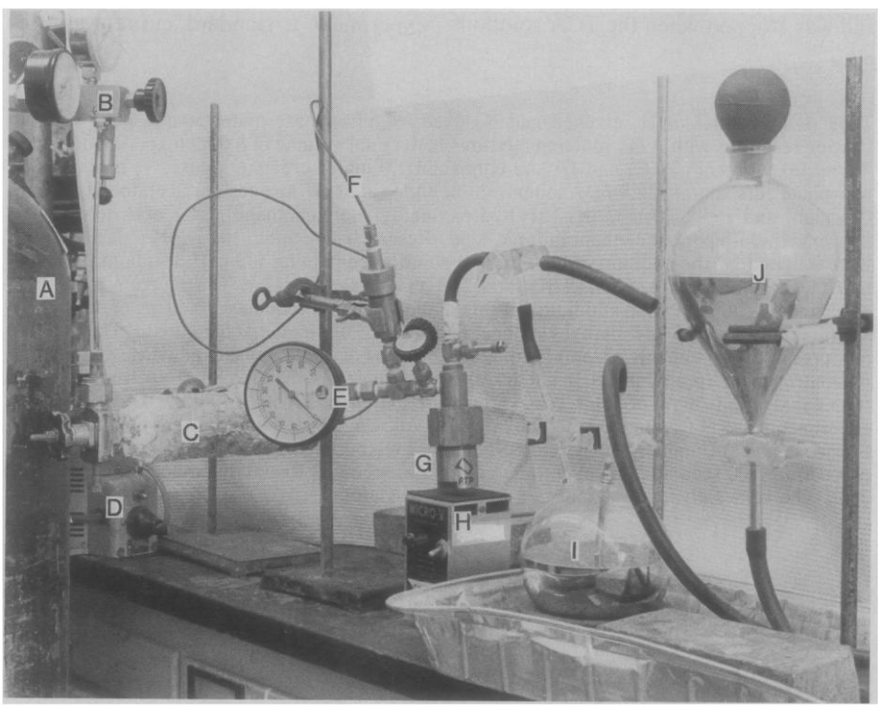

Fig. 1. High-pressure reaction bomb and apparatus built by Sanford Anderson: *A*, tank filled with 70 to 100 atm of high-purity N_2 (Linde); *B*, regulatory valve; *C*, 160°C BASF catalyst in a lecture bottle wrapped with asbestos and Nichrome wire; *D*, variable transformer for adjusting the temperature of the BASF catalyst; *E*, 55-atm Parr gauge; *F*, copper introductory line; *G*, reaction bomb; *H*, magnetic stirrer; *I*, glass vessel filled with mercury for the collection of reaction product gases; and *J*, glass vessel for the collection of displaced mercury.

threaded, removable, stainless steel top. The seal between the top and the vessel was made with a 24-karat-gold gasket (plastic or elastomer gaskets absorb gases at high pressures). Near the top, inside the reaction bomb, two 1-ml glass beakers were cemented with a minimal quantity of epoxy cement. The beakers tilted in opposite directions so that the contents of each beaker could be poured selectively by tilting the vessel in the appropriate direction.

First 0.30 ml of 138 m*M* magnesium ATP was added to the start beaker and then 0.30 ml of trichloroacetic acid (TCA) to the stop beaker under a N_2 atmosphere in an anaerobic glove box. After the nitrogenase reaction mixture had been added to the reaction vessel, the top was screwed on tightly and the bomb was filled with 50 to 51 atm of high-purity N_2 (Linde) that had passed slowly over 160°C BASF (Badische Anilin-und-Soda-Fabrik) R3-11 catalyst (Chemical Dynamics Corporation) to reduce the concentration of contaminating O_2. The pressurized mixture was stirred with a glass-coated magnetic stirring bar for 15 minutes to allow equilibration of N_2 between the gas and liquid phases, and, after the inlet valves on the bomb had been closed, the system was checked for leaks. At time zero the vessel was tipped to add magnesium ATP from the start beaker. The reaction continued for 500 or 800 seconds with constant stirring and was stopped when the TCA solution was tipped from the stop beaker into the stirred reaction mixture. We terminated any reaction occurring from residues in the start beaker immediately thereafter by tipping the acidified reaction mixture into the start beaker. The reaction thus was initiated and terminated under 50 to 51 atm N_2. After TCA addition, the *p*H of the terminated reaction mixture was 3.48 to 4.6. In a control experiment, a piece of 316 stainless steel was placed in a closed vessel and immersed in a similar reaction mixture adjusted with TCA to *p*H 3.35. No H_2 was evolved during 2 hours of incubation as measured with a Clark-type hydrogen electrode (Yellow Springs Instruments).

After termination of the reaction, the gas phase was collected in a glass vessel over purified mercury. After equilibration at atmospheric pressure for 15 minutes with constant stirring of the reaction mixture, we determined the volume of the gas phase by measuring the volume of mercury displaced. To a control (for background NH_3) portion of the enzyme reaction mixture that had not been placed in the reaction vessel, first TCA and then ATP were added to achieve concentrations similar to those of the terminated high-pressure reaction mixture. Microdiffusion (*14*) then was conducted on five replicate portions of the experimental and control solutions, and NH_3 was determined by the method of Chaykin (*15*). Concurrently with each experiment a standard curve was prepared at each of six NH_4Cl concentrations from samples microdiffused in duplicate. The H_2 was determined in the gas phase with a Varian MAT 250 isotope-ratio mass spectrometer by comparing with known H_2 in N_2 standards. Eight concentrations of H_2 were used to prepare standard curves for each experiment, and these curves were linear with added H_2. The detection limit was approximately 0.1 nmole of H_2 in 1.0 ml of sample injected into the mass spectrometer. The quantities of NH_3 and H_2 formed were determined from standard curves established by linear regression analysis. All gas volumes and standards were corrected to the barometric pressure at the time of the experiments.

The quantities of H_2 evolved and N_2 fixed in four experiments at 50- to 51-atm N_2 are presented in Table 1. These results clearly show that, at saturating electron flux, nitrogen at 375 times its reported K_m (*8*) cannot block evolution of H_2 completely; rather, the micromoles of H_2 evolved were approximately equal to the micromoles of N_2 fixed in every experiment. The average molar ratio of H_2 evolved to N_2 fixed was 1.13 ± 0.13. If one assumes that two electrons are required to produce each H_2 and six electrons are needed to reduce one N_2 to two NH_3, then in our experiments H_2 evolution utilizes 27.3 ± 2.4 percent of the electron flux through dinitrogenase at saturating electron flux and 50-atm N_2. This observation is close to the theoretical prediction that 25 percent of the electron flux will be allocated to H_2 evolution at infinite pN_2 if the limiting stoichiometry of H_2 evolved to N_2 fixed is 1:1. Our observations also are in general agreement with the prediction of Rivera-Ortiz and Burris (*8*) from extrapolation of their data that 13 to 23 percent of the total electron flux will be directed toward evolution of H_2 at infinite pN_2. That the ratio of H_2 evolved to N_2 fixed is close to 1:1 suggests that evolution of H_2 is obligatory in the fixation of N_2 by nitrogenase.

Table 1. Quantities of H_2 evolved and N_2 fixed by nitrogenase under 50-atm pN_2. Prior to starting reactions with ATP, reaction mixtures had a total volume of 8.0 ml (experiment A) and 7.7 ml (experiments B, C, and D) and contained 1.0 mg of creatine kinase, 31 m*M* creatine phosphate (disodium), 20.8 m*M* sodium dithionite, 10.4 m*M* magnesium acetate (Matheson, Coleman, and Bell), 20 m*M* tris [tris(hydroxymethyl)aminomethane], 24.1 m*M* hepes [4-(2-hydroxyethyl-l-piperazine-ethanesulfonic acid, Research Organics, Inc.], *p*H 7.1. Reactions were initiated by the addition of 0.30 ml of a solution containing 138 m*M* disodium ATP, 144 m*M* magnesium acetate, 20 m*M* tris, and 24.1 m*M* hepes, *p*H 7.1. The reaction mixture in experiment A contained, in addition, 2.04 mg of dinitrogenase reductase, 0.40 mg of dinitrogenase (preparation A), 5 μ*M* methyl viologen, and 181 μ*M* *A. vinelandii* flavodoxin. The reaction mixtures in experiments B, C, and D contained 5.07 mg of dinitrogenase reductase and 1.08 mg of dinitrogenase (preparation B) but no methyl viologen or flavodoxin. Experiment A was terminated with 0.30 ml of 2.0*M* TCA (Amend), experiment B with 0.30 ml of 4.8*M* TCA, and experiments C and D with 0.30 ml of 6.1*M* TCA. All reagents and buffers were purchased from Sigma Chemical Company unless stated otherwise. Experiments A, B, and C were allowed to proceed for 500 seconds, and experiment D for 800 seconds. Reaction temperature was 23° to 26°C. The values given are averages ± standard deviations.

Experiment	pN_2 (atm)	Specific activity of dinitrogenase (nmole electron pairs min^{-1} mg^{-1} dinitrogenase)	N_2 fixed (μmole)	H_2 evolved (μmole)	H_2/N_2 molar ratio	Percentage of total electron flux allocated to H_2
A	51.0	1648	1.44	1.38	0.958	24.2
B	50.6	2075	4.40	5.45	1.24	29.2
C	50.9	1923	4.24	4.57	1.08	26.4
D	50.9	1808	6.14	7.58	1.23	29.2
				Averages	1.13 ± 0.13	27.3 ± 2.4

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Growth Stasis and Limited Shell Calcification in Larvae of *Cymatium parthenopeum* During Trans-Atlantic Transport

Abstract. *Larvae of the shallow-water marine gastropod* Cymatium parthenopeum *show no appreciable shell calcification and no demonstrable growth as they disperse across the Atlantic Ocean. Evidence of what appears to be physiological specialization for prolonged delay of metamorphosis was found in larvae of this prosobranch gastropod.*

The larvae of at least 19 families of shallow-water prosobranch gastropods can be found in the open waters of the Atlantic and Pacific oceans (*1–4*). In the absence of the environmental cues to initiate metamorphosis, these long-distance (teleplanic) larvae (*2*) may remain planktonic for many months after first becoming competent to metamorphose (*2*). Such flexibility in the timing of metamorphosis permits widespread larval dispersal and may result in virtual panmixia within North Atlantic populations of species such as *Cymatium parthenopeum* (*5, 6*) (Fig. 1). In contrast, larvae of most invertebrate species that have been reared in the laboratory rarely delay metamorphosis for more than a few weeks, most individuals either dying or metamorphosing spontaneously after that time (*1, 7–12*).

The greater potential for delayed metamorphosis and for widespread dispersal of teleplanic larvae is presumed to have a physiological basis (*13*). In particular, metabolism of teleplanic larvae is thought to shift from a growth phase to an equilibrium condition of no-growth energy balance at the time the larvae become competent to metamorphose (*13*), and the shells of some teleplanic larvae in the family Cymatiidae are thought to be uncalcified (*2, 4*). These characteristics are regarded as adaptive because they would discourage weight gain during transoceanic transport (*13*). However, there has been no documentation of either reduced growth or reduced calcification in teleplanic larvae. For the few nonteleplanic prosobranch species that have been studied in the laboratory (*Crepidula fornicata* and *C. plana*), larval growth (size, weight, protein content) continues unabated during the period of delayed metamorphosis, and larval shells are calcified (*11, 14, 15*). Growth stasis has been shown only for larvae of opisthobranch gastropods (*16, 17*).

An inverse relation between rate of development and ability to postpone metamorphosis has recently been shown for larvae of *Crepidula fornicata* and *C. plana* (*14, 15*). It appears that the maximum length of time that metamorphosis can be delayed by feeding (planktotrophic) larvae may be related to the rate at which such larvae develop toward a genetically determined end for maintaining larval form and function (*11, 14*). Because the presumed cessation of growth by teleplanic larvae may be related to their ability to delay metamorphosis, it is important to know whether their growth actually does cease during transoceanic transport. We report that teleplanic larvae of the prosobranch gastropod *Cymatium parthenopeum* are physiologically different from species with a more restricted range and a shorter maximum planktonic duration. In particular, we show that larvae of *C. parthenopeum* do not grow during transport across the Atlantic Ocean and that the larval shells are not significantly calcified.

Larvae of *C. parthenopeum* were obtained from plankton tows made between 1964 and 1966 along a west-east transect (Fig. 1). Larvae were killed in buffered Formalin (pH ~ 8.0) and later transferred to 80 percent ethanol buffered to pH ~ 8.0 with sodium borate. Other larvae were obtained from coastal plankton samples (13°21′N, 61°28′W) collected in 1982 by S. Humphries and preserved in Formalin buffered to a pH of approximately 8.0 with sodium borate. Larval shell lengths were measured with a dissecting microscope (×40) equipped with an ocular micrometer. Total dry weights (n = 150) and ash-free dry weights (n = 53) of individual larvae were then determined by standard techniques (*11, 18*). In addition, empty shells of eight individuals that had died and decomposed after being cultured for up to several months after their collection were dried, weighed, and reduced to ash in order to look at ash content of the shell. For comparison, total dry weight and ash content were determined for competent larvae (approximately 1200 μm in shell length) of nonteleplanic prosobranch species *Crepidula fornicata* and *C. plana*.

The mean ash content of intact teleplanic larvae was 4.8 percent [standard error of the mean (S.E.M.) = 0.3 percent, n = 38] for larvae preserved in alcohol and 6.5 percent (S.E.M. = 0.4 percent, n = 15) for those preserved in buffered Formalin. These differences are small but statistically significant

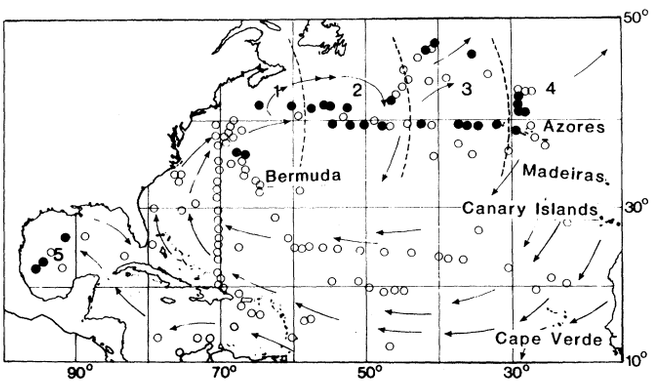

Fig. 1. Distribution of veliger larvae of *Cymatium parthenopeum* in the North Atlantic Ocean. Closed circles denote sites where larvae were obtained; open circles show other locations where larvae of this species have been found. Zones 1 through 4 (see Table 1) are delineated by dashed lines. Arrows indicate surface circulation of the North Atlantic gyre.