Interactions among N_2, N_2O, and C_2H_2 as Substrates and Inhibitors of Nitrogenase from *Azotobacter vinelandii*†

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ABSTRACT: An isotope-ratio mass spectrometer and ^{15}N-enriched N_2O have been used to study the interactions of N_2O with other substrates of nitrogenase under such conditions that the concentrations of MgATP and sodium dithionite and the ratio of dinitrogenase reductase to dinitrogenase were high enough to favor substrate reduction over H_2 evolution. The results have shown that N_2 is competitive with N_2O and N_2O is competitive with C_2H_2, whereas C_2H_2 is noncompetitive with N_2O. The K_{is}'s for N_2, N_2O, and C_2H_2 are 0.37, 0.50, and 0.014 atm, respectively. The K_{ii} for C_2H_2 is 0.016 atm. N_2O at a pressure of 40 atm completely inhibited H_2 formation. Whereas N_2O is a noncompetitive inhibitor of HD formation, D_2 did not exert any effect on N_2O reduction. At constant pN_2, an increase in pN_2O decreased both NH_3 and H_2 production. At fixed pN_2O, an increase in pN_2 restored NH_3 production, but changes in H_2 evolution and NH_3 production were different; at low pN_2O (0, 0.1, 0.2 atm), H_2 evolution declined with rising pN_2; however, at high pN_2O (1.0, 2.0 atm), the rate of H_2 evolution was corestored with the rate of NH_3 production by increasing pN_2. A mechanism has been suggested to explain the experimental observations.

Nitrogenase is composed of two electron transferring proteins, dinitrogenase (MoFe protein) and dinitrogenase reductase (Fe protein) (Hageman & Burris, 1980). Under physiological conditions the dinitrogenase reductase is reduced by ferredoxin or flavodoxin (Mortenson, 1963, 1964; Shethna, 1966). Dithionite is often used as the reductant for this protein in in vitro systems. Reduced dinitrogenase reductase transfers a single electron at a time to dinitrogenase with the concomitant hydrolysis of two molecules of MgATP to produce two molecules of MgADP plus inorganic phosphate (Tso & Burris, 1973). Dinitrogenase, with its multiple iron-sulfur centers and its FeMoco prosthetic groups, serves as a electron sink capable of reducing all of the substrates of nitrogenase. In addition to reducing its physiological substrate N_2 to NH_3, nitrogenase is also capable of reducing a variety of low molecular weight compounds such as nitrous oxide, acetylene, azide, cyanide, methyl isocyanide, cyclopropene, protons, and analogues of some of these compounds (Hardy, 1979; Postgate, 1982). All substrates compete for electrons from the reduced dinitrogenase; therefore, they are mutually inhibitory (Burris, 1979). N_2 is a competitive inhibitor of C_2H_2 reduction, whereas C_2H_2 is a noncompetitive inhibitor of N_2 fixation (Rivera-Ortiz & Burris, 1975). This may be the only known example in enzymology that two substrates have a nonreciprocal inhibitory pattern.

Reduction of N_2O was the first reported example of reduction of a substrate other than N_2 by nitrogenase; cultures of *Azotobacter vinelandii* and soybean nodules assimilated ^{15}N from an atmosphere containing $^{15}N_2O$ (Mozen & Burris, 1954). N_2O reduction to N_2 plus H_2O involves the transfer of two reducing equivalents and two protons, and N_2 in turn is further reduced to NH_3 (Hoch et al., 1960; Hardy & Knight, 1966; Jensen & Burris, 1986). N_2O is one of the only two demonstrated competitive inhibitors of N_2 reduction (H_2 being the other) (Repaski & Wilson, 1952; Rivera-Ortiz & Burris, 1975; Jensen & Burris, 1986). In contrast to N_2, which is a competitive inhibitor of C_2H_2 reduction and is unable to suppress nitrogenase-mediated H_2 evolution even at infinitely high pN_2 (Rivera-Ortiz & Burris, 1975; Simpson & Burris, 1984), N_2O has been reported to be a noncompetitive inhibitor of C_2H_2 reduction (Rivera-Ortiz & Burris, 1975) and is predicted by extrapolation of the experimental data to wipe out H_2 evolution at infinite pN_2O (Jensen & Burris, 1986). Also in contrast to N_2 reduction which is inhibited by H_2 (Wilson & Umbreit, 1937; Guth & Burris, 1983), N_2O reduction has been reported to be insensitive to H_2 inhibition (Hoch et al., 1960; Jensen & Burris, 1986).

The stoichiometry of one H_2 evolved per N_2 fixed under 50 atm of N_2 and saturating electron flux indicates that H_2 evolution is an inherent property of the N_2 fixation reaction of nitrogenase (Simpson & Burris, 1984). Several investigators have suggested mechanisms to explain this phenomenon (Chatt, 1980; Guth & Burris, 1983; Lowe & Thorneley, 1984a,b). It has been proposed that N_2 binds to a specific redox form of the enzyme that contains metal-bound hydrides and displaces two hydrides to form H_2. Under a high pN_2 and saturating electron flux, it is suggested that electrons that otherwise would reduce protons (so-called electron leakage) would be redirected to reduce N_2 and would produce one H_2 per N_2 fixed. Direct experimental proof of this proposal still is lacking.

In the present paper we have used $^{15}N_2O$ to study the effect of N_2 on N_2O reduction and the interactions between N_2O and C_2H_2. We also have placed nitrogenase under high pN_2O, up to 40 atm, to ascertain the effect of pN_2O on H_2 evolution. We describe the utilization of N_2O as a probe to investigate the proposed mechanism for H_2 production via H_2 displacement by N_2.

Materials and Methods

Growth of Bacteria and Purification of Nitrogenase. *A. vinelandii* OP was grown at 30 °C in a 300-L fermenter on the nitrogen-free medium described by Strandberg and Wilson (1968). The nitrogenase proteins were prepared from frozen cell paste in two batches by the method of Hageman and

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Burris (1980) to specific activities of 1256–1898 and 897–1826 nmol of ethylene produced (minute·mg of protein) for dinitrogenase and dinitrogenase reductase, respectively. The proteins were homogeneous as indicated by denaturing polyacrylamide gel electrophoresis.

Preparation of $^{15}N_2O$. Isotopically labeled nitrous oxide was prepared by the decomposition of $^{15}NH_4NO_3$ [prepared from 73 atom % ^{15}N $(^{15}NH_4)_2SO_4$ and HNO_3; Burris & Wilson, 1957]. The $^{15}NH_4NO_3$ was heat decomposed into N_2O and water by a modified procedure of Friedman and Bigeleisen (1950). To prevent sublimation of NH_4NO_3 to cooler portions of the reaction tube, the decomposition was carried out under 1 atm of helium rather than under high vacuum. A round-bottom flask containing anhydrous $^{15}NH_4NO_3$ was placed vertically in an electrically heated tube and heated to 220–240 °C. The N_2O formed was collected in a glass vessel over mercury. At the conclusion of the reaction (overnight), the N_2O was passed through an alcohol–dry ice trap to remove higher nitrogen oxides and then was condensed in a U-shaped tube immersed in a slurry of solid ethanol in liquid ethanol (−117.3 °C). Possible contaminant N_2, O_2, and NO were removed by evacuation. N_2O was collected by warming the U-tube. This purification procedure was repeated three times. The preparation contained the molecular species of N_2O ($^{15}N^{14}NO$,$^{14}N^{15}NO$,$^{15}N^{15}NO$,$^{14}N^{14}NO$) with 35.7 atom % ^{15}N determined by mass spectrometric analysis. Elsewhere in this paper the terms $^{15}N_2O$ and $^{15}N_2$ are used in the generic sense to indicate ^{15}N-labeled N_2O and N_2 rather than to indicate the specific molecular species $^{15}N^{15}NO$ and $^{15}N^{15}N$.

Nitrogenase Assays. Unless otherwise stated, reactions were carried out at 30 °C with shaking. The reaction mixture contained the following in 1 mL: 5.91 μmol of ATP (Sigma Chemical Co.), 11.07 μmol of magnesium acetate (Matheson Coleman and Bell), 27.9 μmol of creatine phosphate (United States Biochemical Co.), 0.1 mg of creatine phosphokinase (EC 2.7.3.2; Sigma Chemical Co.), 20 μmol of $Na_2S_2O_4$ (Sigma Chemical Co.), 20 μmol of tris(hydroxymethyl)-aminomethane (Tris; Boehringer Mannheim Biochemicals), 50 μmol of *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid (Hepes; Research Organics Inc.) adjusted to pH 7.5, and nitrogenase proteins as indicated.

High-Pressure Experiments. The high-pressure experiments were conducted in the way described previously by Simpson and Burris (1984). Assay mixtures contained the following in 1 mL: 27.87 μmol of creatine phosphate, 0.1 mg of creatine phosphokinase, 14.9 μmol of $Na_2S_2O_4$, 15.4 μmol of magnesium acetate, 5.92 μmol of ATP, 20 μmol of Tris, 50 μmol of Hepes, adjusted to pH 7.5, and nitrogenase proteins as indicated. The reaction vessel was a 22.2-mL stainless steel chamber. Two 1-mL beakers, which tilted in opposite directions, were glued with a minimal amount of epoxy cement to the inner wall of the vessel above the enzyme solution level. In a N_2-filled anaerobic glovebox, 0.2 mL of MgATP, 0.2 mL of 12.2 M TCA, and 7.8 mL of enzyme solution were transferred into the two 1-mL beakers and the stainless steel cup, respectively; a gold gasket was placed on the top of the cup, the cap was screwed on tightly, and the reaction vessel was removed from the glovebox. The reaction bomb was filled with 1.5 atm of N_2O (measured with a mercury manometer) and then with 50 atm of N_2 (measured with a 55-atm Parr pressure gauge) in some experiments, or just with 40 atm of N_2O in other experiments. To make sure that the flow of gases always was into and never out of the reaction bomb when we were filling with gases to high pressure, we carefully controlled the valves so that the pressure in the inlet tubing outside the bomb always was higher than the pressure inside the bomb. The enzyme solution was stirred with a glass-coated magnetic stirring bar for 5 min to allow equilibration of the gases with the liquid phase. The reaction was initiated by tipping MgATP into the enzyme solution. Reactions were conducted at room temperature (about 22 °C) for 10 min with stirring adequate to give maximal rates, and reactions were terminated by tipping TCA into the vessels. The gas in the bomb was released slowly and was collected in a glass reservoir by displacement of mercury. With the reservoir still connected to the reaction bomb, the reaction solution was stirred for several minutes at atmospheric pressure to allow equilibration of dissolved gases between liquid and gas phases. Then the volume of displaced mercury was measured to permit calculation of the total amount of H_2 produced and the final pressure in the reaction vessel. Atmospheric pressure was measured at the time of collection of the product gases. The TCA-acidified reaction mixture was stored frozen at −20 °C.

C_2H_2 Reduction. The C_2H_2 reduction assays were performed in 5-mL bottles fitted with vaccine stoppers and filled with 1 atm of argon and 0.2 mL of enzyme mixture. Different amounts of substrate (C_2H_2, generated by addition of CaC_2 to H_2O) and inhibitor (N_2O, USP, purchased from Badger Welding Supplies, Madison, WI, and purified by condensing in a slurry of solid acetone in liquid acetone, −95 °C) were added to the reaction bottles with gas-tight syringes. Assays were initiated by addition of MgATP and were terminated by addition of 50 μL of 25% trichloroacetic acid. Gas samples (0.5 mL) were removed with 1.0-mL plastic syringes, and ethylene was measured on a Carle 9500 gas chromatographic unit equipped with a flame ionization detector and a column of Porapak R operated at 75 °C with N_2 as the carrier gas.

N_2O Reduction. Assays for N_2O reduction were conducted in the same manner as for C_2H_2 reduction except that helium was used in place of argon. N_2O (with or without ^{15}N enrichment) was the substrate, and N_2, C_2H_2, or D_2 was the inhibitor. $^{15}N_2$ production from $^{15}N_2O$ was analyzed on an MAT 250 isotope-ratio mass spectrometer. To optimize the sample size for analysis, a measured amount of tank N_2 as indicated was added to each reaction bottle on termination of the reaction. Because $^{15}N_2O$ ionizes in the mass spectrometer to produce major peaks at masses 28, 29, 30, 44, and 45, measurement of $^{15}N_2$ production from $^{15}N_2O$ is impractical unless N_2 (including $^{15}N_2$) is separated from bulk $^{15}N_2O$. We accomplished this by freezing the N_2O by immersing the reaction bottle in liquid nitrogen for 2 min. The reaction bottle was connected to the sample inlet port of the mass spectrometer with the device illustrated and described in Figure 1. The mass 29 and mass 28 signals were compared, and their ratio was indicated on the printout from the mass spectrometer. An air standard was analyzed in parallel with each sample on the standard side inlet of the mass spectrometer. The production of $^{15}N_2$ was expressed as the excess of the ratio of mass 29/28 above the ratio of the standard (air): v = 29/28(sample) − 29/28(air standard).

Purification of N_2O and C_2H_2. N_2O was transferred from a 100-ft^3 tank to a lecture bottle where it was condensed by immersing the lecture bottle in a liquid–solid acetone bath (−95 °C). Any contaminating N_2, O_2, and NO were removed by evacuation of gases above the condensed N_2O. Upon warming, N_2O in the lecture bottle had a pressure of 600 psi. C_2H_2 was generated with CaC_2 and distilled water. To minimize O_2 contamination in the C_2H_2, we added a small amount of dithionite to the water and used an excess of CaC_2 so excess C_2H_2 purged the water in the displacement reservoir.

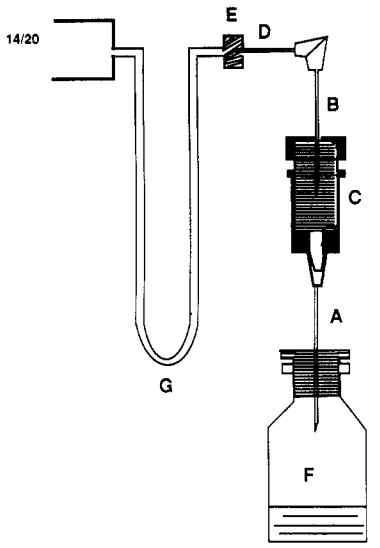


FIGURE 1: Device for introducing gas into a mass spectrometer through a 14/20 standard taper joint. Needle A penetrates the vaccine stopper of the reaction vessel before the vessel is immersed in liquid nitrogen (after immersion the stopper is too hard to permit penetration). Needle B, part of a double-needle unit, enters about halfway into silicone rubber plug C (1–1.5 cm long). Needle D of the double needle penetrates septum E and is pumped out with the evacuation system of the mass spectrometer. A gas sample from reaction bottle F now can be introduced to the mass spectrometer by pushing needle B all the way through silicone rubber plug C. The gas passes through metal U-tube G immersed in liquid N_2. Double needles can be produced by cutting the plastic hubs of two needles at a 45° angle and joining these hubs after heating them over a candle. Deep septa can be cut from silicone rubber stoppers.

H_2 Production. For experiments in which a relatively high production of H_2 was anticipated, H_2 was determined in a 0.5-mL gas sample by gas chromatography with a thermal conductivity detector and a column of molecular sieve 5A. Argon was used as carrier gas, and the column temperature was 50 °C. The instrument was calibrated with known amounts of H_2 in argon. In experiments (such as high-pressure experiments) in which low levels of H_2 were expected, H_2 was analyzed in 0.5-mL gas samples with the MAT 250 isotope-ratio mass spectrometer. Standard curves for H_2 were prepared by analyzing standards of H_2 in N_2, H_2 in N_2O, or H_2 in N_2 plus N_2O.

Ammonia Production. The reaction mixture was centrifuged, and a sample of the supernatant was transferred to a clean 9-mL bottle to which 1 mL of 4.5 M K_2CO_3 was added to initiate the microdiffusion of NH_3 to a glass rod previously dipped in 1 M H_2SO_4 (Burris, 1972). After 4-h microdiffusion, NH_3 was assayed by the indophenol method of Chaykin (1969). A_{625} was measured after incubation with the indophenol reagent at room temperature for 1 h. NH_4Cl was used as the standard.

Treatment of Kinetic Data. Kinetic data were analyzed by the computer program described by Cleland (1963, 1967). The initial inhibition patterns, which were helpful for choosing the appropriate rate equations for computer analysis, were obtained from double-reciprocal plots (1/velocity vs 1/substrate concentration). Data showing competitive and noncompetitive inhibition were fitted to the equations $v = VA/[K(1 + I/K_i) + A]$ and $v = VA[K(1 + I/K_{is}) + A(1 + I/K_{ii})]$, respectively, where A = substrate concentration, I = inhibitor concentration, v = velocity, V = maximum velocity, and K = the inhibition constant. Experimental data points (usually the average of two or three observations) were recorded and the plotted lines were calculated by the computer program that gave greatest weight to the highest velocities and least weight to the lowest velocities. Data points in parentheses were not included in the computer treatment.

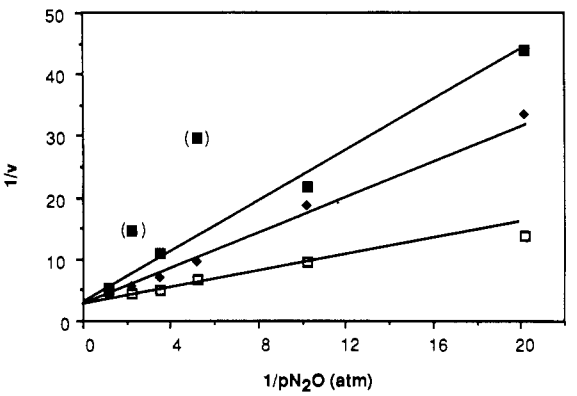


FIGURE 2: Inhibition of N_2O reduction by N_2. The reaction mixture contained the following in 1 mL: 0.138 mg of dinitrogenase, 0.474 mg of dinitrogenase reductase, 5.91 μmol of ATP, 11.07 μmol of magnesium acetate, 27.9 μmol of creatine phosphate, 0.1 mg of creatine phosphokinase, 20 μmol of $Na_2S_2O_4$, 20 μmol of Tris, and 50 μmol of Hepes (pH 7.5, adjusted with HCl). Reactions were conducted in 5-mL bottles containing 0.2 mL of enzyme mixture, 1 atm of He, varying $p^{15}N_2O$ (35 atom % ^{15}N) as indicated, and pN_2 at 0 atm (□), 0.4 atm (◆), and 0.8 atm (■) at 30 °C for 30 min. Reaction was initiated by addition of MgATP and stopped with 50 μL of 25% TCA. v = the ratio of mass 29/28 of the samples minus the ratio of 29/28 of the air standard [v = 29/28(sample) − 29/28(air)].

RESULTS

Effect of N_2 on N_2O Reduction. N_2 is not only an inhibitor of but also one of the products of N_2O reduction, so this poses difficulties in studying the effect of N_2 on N_2O reduction. To distinguish product N_2 from inhibitor N_2, we utilized $^{15}N_2O$ as the substrate, so that we were able to measure the $^{15}N_2$ production with an isotope-ratio mass spectrometer. Under our conditions of measurement, $^{15}N_2O$ breaks down in the electron beam of the mass spectrometer to produce $^{15}N_2$. As this $^{15}N_2$ would obscure the measurement of enzymatically produced $^{15}N_2$, we immersed the reaction bottles in liquid N_2 before introducing gas samples from them into the mass spectrometer; this effectively removed $^{15}N_2O$ so that it did not enter the mass spectrometer. A series of bottles was filled with varying amounts of $^{15}N_2O$ (from 0 to 0.5 atm) and 0.8 atm of N_2, and these gas mixtures were analyzed for mass 29 and mass 28 peaks. The results (not shown) indicated that N_2O was effectively removed by the liquid N_2, as there was no measurable difference between the ratio of mass 29 to mass 28 of samples and standards (air) throughout the concentration range of $^{15}N_2O$ used. A time course of $^{15}N_2O$ reduction by purified nitrogenase from *A. vinelandii* was run as described under Materials and Methods. The rate of $^{15}N_2$ production was linear up to 35 min after initiation of the reaction (data not shown). As the analytical methods were reliable, we were able to investigate the effect of N_2 on N_2O reduction by including varying amounts of $^{15}N_2O$ and fixed levels of N_2 in the reaction bottles and examining the ratio of mass 29 to mass 28 of the product gas. Figure 2 records a double-reciprocal plot of one of the experiments. Another experiment gave similar results (data not shown). Computer analysis of the data indicated that N_2 is a competitive inhibitor of N_2O reduction with a K_{is} of 0.37 atm (N_2). The K_m for N_2O was estimated to be 0.23 atm, which is comparable to 0.24 atm for nitrogenase from *Klebsiella pneumoniae*, reported by Jensen and Burris (1986).

Interactions between N_2O and C_2H_2. Figure 3 records a double-reciprocal plot showing that C_2H_2 noncompetitively inhibited N_2O reduction. The kinetic constants obtained by

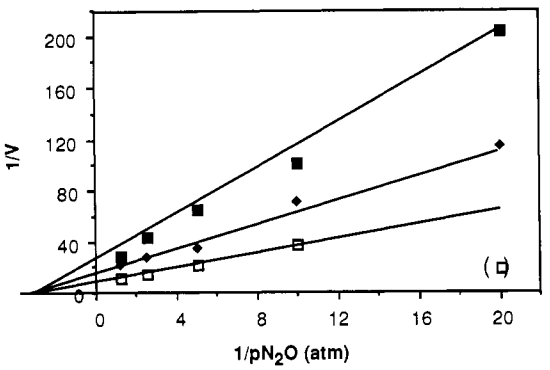


FIGURE 3: C_2H_2 inhibition of N_2O reduction. Experimental details were as described in the legend to Figure 2 and under Materials and Methods except that the reaction was run for 20 min and the reaction mixture contained the following in 1 mL: 0.495 mg of dinitrogenase reductase and 0.103 mg of dinitrogenase. (□) C_2H_2 = 0 atm; (◆) C_2H_2 = 0.01 atm; (■) C_2H_2 = 0.03 atm. v = 29/28(sample) − 29/28(air).

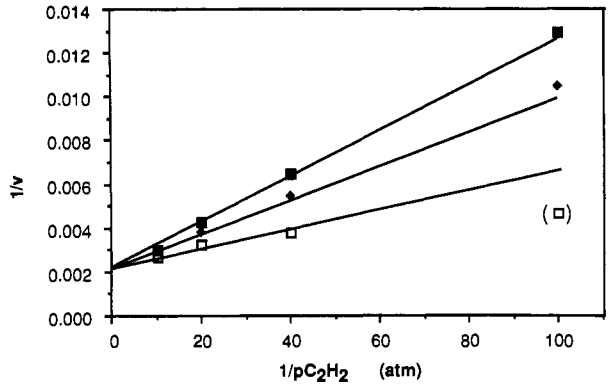


FIGURE 4: N_2O inhibition of C_2H_2 reduction. Experimental details were as described in the legend to Figure 2 and under Materials and Methods except that the reaction time was 15 min. (□) N_2O = 0 atm; (◆) N_2O = 0.3 atm; (■) N_2O = 0.6 atm. v = nmol of C_2H_4/15 min.

computer calculation are K_{is} = 0.014 atm and K_{ii} = 0.016 atm. The N_2O inhibition of C_2H_2 reduction has been reported by Rivera-Ortiz and Burris (1975) to be noncompetitive. Knowing that N_2 and N_2O are mutually competitive inhibitors, that C_2H_2 inhibits both N_2 and N_2O reductions noncompetitively, and that N_2 is a competitive inhibitor of C_2H_2 reduction, we reasoned that N_2O could be a competitive inhibitor of C_2H_2 reduction, contrary to our earlier conclusion. Before reexamining this topic, we purified commercially obtained N_2O gas as described under Materials and Methods. We wanted to be sure that the N_2 was free of NO, a very potent inhibitor of nitrogenase. Results shown in Figure 4 indicate that N_2O is a competitive inhibitor of C_2H_2 reduction; the K_{is} is 0.50 atm. The K_m for C_2H_2 was estimated to be 0.022 atm, which is close to the estimation (0.015 atm) reported by other workers (Hwang et al., 1973).

Interactions between N_2O and H_2. Nitrogen-mediated HD formation occurs only in the presence of N_2 (Li & Burris, 1983). The competition between HD formation and N_2 reduction for electrons from the same electron pool has been suggested as the mechanistic basis for competitive H_2 inhibition of N_2 reduction (Guth & Burris, 1983). N_2O does not support, but noncompetitively inhibits, HD formation by nitrogenase, whereas H_2 alone does not exert any effect on N_2O reduction (Jensen & Burris, 1986). We tested the effect of D_2 on N_2O reduction in the presence of 0.1 atm of N_2 (higher pN_2 would inhibit HD formation; Li & Burris, 1983). The results are not shown, but at pD_2 levels of 0.1, 0.2, and 0.4 atm there was

Table I: Inhibition of H_2 Evolution by N_2O[a]

pN_2O (atm)	nmol of H_2/ (bottle·15 min)	nmol of NH_3/ (bottle·15 min)	total electron pairs/(mg of dinitrogenase·min)	%
0[b]	630.8	1044.0	2202.4	100.0
1	235.3	3.3	240.3	10.9
2	93.4	ND[c]	93.4	4.2
3	25.3	ND	25.3	1.1
4	58.9	ND	58.9	2.7
40	ND	ND	0	0

[a] The reaction mixture contained the following in 1 mL: 0.127 mg of dinitrogenase, 0.703 mg of dinitrogenase reductase, 6.15 μmol of ATP, 15.95 μmol of magnesium acetate, 28.9 μmol of creatine phosphate, 0.1 mg of creatine phosphokinase, 20.76 μmol of $Na_2S_2O_4$, 20 μmol of Tris, and 50 μmol of Hepes (pH 7.5, adjusted with HCl). Reactions under 0–4 atm of N_2O were run in 9-mL bottles containing 0.5 mL of enzyme mixture. Reactions were initiated by addition of MgATP, run for 15 min at 30 °C, and terminated with 13 μL of 12.2 M TCA. Reaction under 40 atm of N_2O was performed in a stainless steel bomb as described. Total activity was the sum of electrons allocated to H_2 and NH_3. [b] 1 atm of N_2. [c] ND, not detectable.

no change in the profiles of the N_2O titration curve, as measured by the ratio of mass 29 to mass 28. HD formation was not analyzed in the experiment, as the sample size was limited.

Although H_2 did not affect N_2O reduction, N_2O strongly inhibited H_2 evolution by nitrogenase. Table I records the experiments in which H_2 evolution was quantitated at high pN_2O, up to 40 atm. The total specific activity was determined to be 2105 nmol of electron pairs/(mg of dinitrogenase protein·min) in a control experiment run under the same conditions as for the other samples, except that 1 atm of N_2 was used in place of N_2O. This activity is the sum of the number of electron pairs allocated to H_2 and NH_3; we assume 1 pair of electrons directed to H_2 and 1.5 pairs to NH_3. This activity was taken to be 100% and assumes that N_2O does not alter the specific activity of the enzyme (Jensen & Burris, 1986; Guth & Burris, 1983). One atmosphere of N_2O reduced H_2 evolution to 11.4% of the control, and 3 atm of N_2O almost abolished H_2 production by nitrogenase. The enzyme also was placed under 40-atm pN_2O that had been purified as described under Materials and Methods and was passed slowly over 50 °C BASF (Badishce Anilin-und-Soda-Fabrik) R3-11 catalyst (Chemical Dynamics Corp.) to reduce the concentration of contaminating O_2. Under such conditions, neither H_2 nor NH_3 was detectable by the isotope-ratio mass spectrometer (sensitivity for H_2 being 1 nmol of H_2/mL) or colorimetrically (Chaykin, 1969). Two controls of the high-pressure experiment that were run under either 1 atm of N_2O or 1 atm of Ar gave 231 and 2303 nmol of H_2/(mg of dinitrogenase protein·min), respectively, and no NH_3 formation was detected.

Can N_2 Restore H_2 Evolution after N_2O Inhibition? Both N_2 and N_2O inhibit H_2 evolution, but to different extents. We performed an experiment to examine how the pN_2 or pN_2O affects partitioning of electrons to protons (H_2 evolution) when one of these two gases is kept constant and the other is varied. With a constant pN_2 at 0.25, 0.5, 1.0, and 2.0 atm, an increase in pN_2O from 0 to 2 atm always led to a decrease in H_2 evolution, in NH_3 production, and therefore in the total electron flux directed to H_2 plus NH_3 (Figure 5). If the pN_2O remained constant and the pN_2 was changed, however, the curves exhibited different trends in H_2 evolution compared to NH_3 production. With increasing pN_2 from 0.25 to 2.0 atm, NH_3 production increased regardless of the pN_2O, whereas H_2 production decreased at low pN_2O (0, 0.1, 0.2 atm), and increased at high pN_2O (1.0, 2.0 atm) (Figure 6). Data from assays under 2-atm pN_2O were replotted in Figure 7, and this clearly indicates that an increase in the pN_2 restored both NH_3

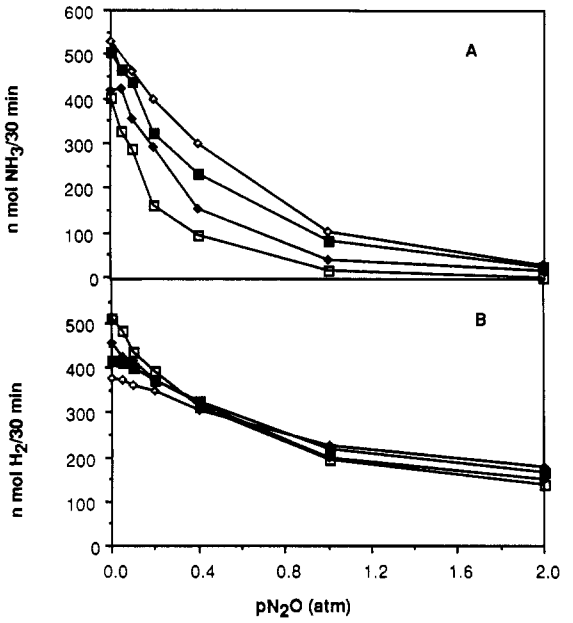


FIGURE 5: Effect of pN_2O on H_2 and NH_3 production. Reactions were performed in 5-mL bottles as described in the legend to Figure 2 and under Materials and Methods except that dinitrogenase and dinitrogenase reductase were 0.177 and 0.916 mg/mL, respectively. H_2 was analyzed on a gas chromatography unit equipped with a thermal conductivity detector, and NH_3 was measured as described. (A) Effect of pN_2O on NH_3 production; (B) effect of pN_2O on H_2 evolution. (□) N_2 = 0.25 atm; (◆) N_2 = 0.5 atm; (■) N_2 = 1.0 atm; (◇) N_2 = 2.0 atm.

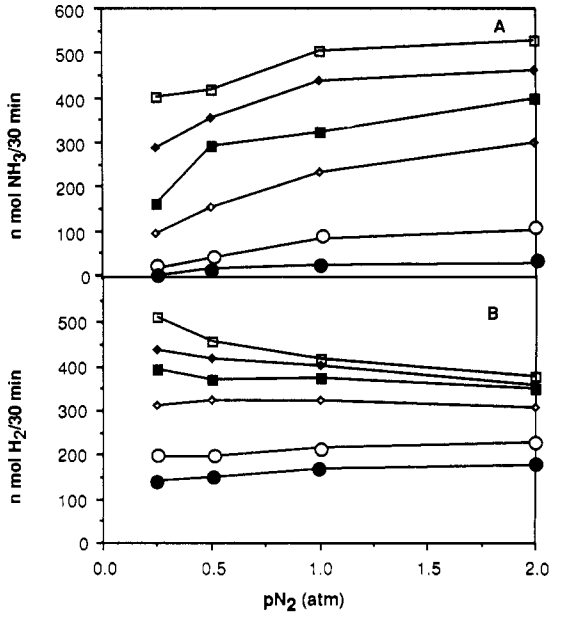


FIGURE 6: Effect of pN_2 on H_2 and NH_3 production. Data were taken from Figure 5. NH_3 production (A) and H_2 evolution (B) were plotted vs pN_2 at pN_2O's of 0 (□), 0.1 (◆), 0.2 (■), 0.4 (◇), 1.0 (○), and 2.0 atm (●).

and H_2 production. This observation was confirmed by an independent study performed with a high-pressure bomb, as described under Materials and Methods, and the results are shown in Figure 8. Under 1-atm pN_2, the total activity (sum of electrons directed to H_2 and NH_3) was 996 electron pairs/(mg of dinitrogenase protein·min); 27.5% went to H_2 and 72.5% went to NH_3. Under 1 atm of N_2 plus 1.5 atm of N_2O, NH_3 production was almost abolished, and the remaining H_2 evolution accounted for about 5% of the total enzyme activity. When the pN_2O was kept constant at 1.5 atm and the pN_2 raised to 50 atm, the activity accounted for by H_2 and

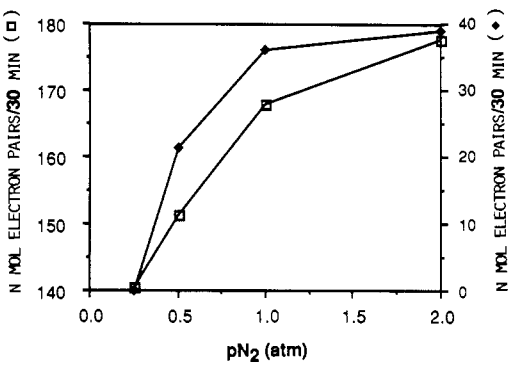


FIGURE 7: Dependence on pN_2 of H_2 and NH_3 production at a pN_2O of 2 atm. Data were taken from Figure 6 and replotted as nmol of electron pairs/30 min vs pN_2. (◆) NH_3; (□) H_2.

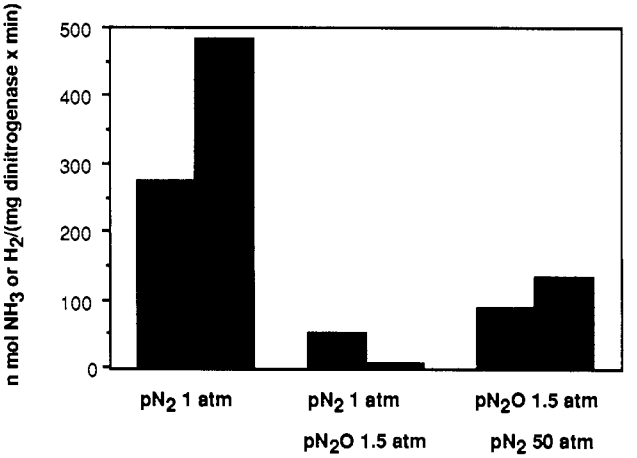


FIGURE 8: Restoration by N_2 of NH_3 and H_2 production from N_2O inhibition. Reaction mixtures were described under Materials and Methods for the high-pressure experiment and in addition contained the following in 1 mL: 0.685 mg of dinitrogenase reductase and 0.128 mg of dinitrogenase. Reactions under 3 atm of total gas pressure were conducted in 9-mL bottles containing 0.5 mL of reaction mixture, 1 atm of N_2, and N_2O as indicated. Reactions, initiated by addition of 13 μL of MgATP and terminated by 13 μL of 12.2 M TCA, were run for 10 min with rapid shaking at room temperature (22 °C). The high-pressure experiment was performed in a stainless steel bomb as described under Materials and Methods. H_2 (left bars) and NH_3 (right bars) from all experiments were analyzed with an isotope-ratio mass spectrometer and an indophenol colorimetric method, respectively, as described.

NH_3 production was about 29% of the total enzymatic activity, and H_2 evolution was corestored with NH_3 production. The ratio of H_2 evolved to N_2 fixed was 1.30/1.

DISCUSSION

Since the first report of N_2O as an alternative substrate for nitrogenase (Mozen & Burris, 1954), considerable information has accumulated on N_2O both as a substrate and as an inhibitor of nitrogenase. N_2O is reduced to H_2O and N_2, and the N_2 in turn is reduced further to NH_3. N_2O reduction, which involves two protons and two reducing equivalents, is competitive with N_2 reduction. Because of the difficulty in distinguishing added N_2 from N_2 produced from N_2O, the effect of N_2 on N_2O has not been determined previously. The fact that N_2 is a competitive inhibitor of C_2H_2, whereas C_2H_2 is a noncompetitive inhibitor of N_2, prompted us to test experimentally whether N_2O and N_2 have a comparable type of interaction. By using ^{15}N-labeled N_2O as substrate and an isotope-ratio mass spectrometer as the analytical device, we have demonstrated that N_2 inhibits N_2O reduction competitively. We also tried to fit the data to a noncompetitive inhibition program, and a result of 0.387 ± 0.076 was obtained

for K_{is}. This result had a greater standard error than that obtained by fitting to a competitive pattern (0.367 ± 0.038). Furthermore, with noncompetitive fitting, the K_{ii} was calculated by computer to be 19.06 ± 61.88; thus the standard error was three times greater than the constant itself. Although an isotope-fractionation factor of 1.039 has been reported for N_2O reduction in intact *A. vinelandii* cells (Yamazaki et al., 1987), the proportion of $^{15}N_2$ in the total product N_2 would not be changed significantly by such fractionation. Measurement of ^{15}N enrichment in the product should faithfully indicate the N_2 production from N_2O. Thus, we are confident that N_2 is competitive vs N_2O. Our estimated value of 0.23 atm of N_2O for the K_m for nitrogenase from *A. vinelandii* is in good agreement with the value of 0.24 atm of N_2O for the K_m of nitrogenase from *K. pneumoniae* (Jensen & Burris, 1986). Our observed K_i of N_2 against N_2O was 0.11 atm; this appears to be the first report of this K_i. Comparing the K_m (N_2O) with the K_i for N_2O against N_2 fixation [reported to be 0.11 atm by Rivera-Ortiz and Burris (1975) and Jensen and Burris (1986)] shows that the K_i is about half the K_m. In contrast, the K_i of N_2 against N_2O is over three times the K_m of N_2 (0.11 atm). So N_2O is a stronger inhibitor of N_2 fixation than is N_2 of N_2O reduction.

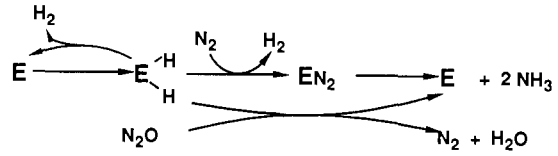
C_2H_2 reduction has received much attention because of its practical use in assaying nitrogenase activity. We have shown that on one hand N_2O is a competitive inhibitor of C_2H_2 reduction, and on the other hand C_2H_2 is a noncompetitive inhibitor of N_2O reduction. A comparable response holds true for N_2 vs C_2H_2 (Rivera-Ortiz & Burris, 1975). Rivera-Ortiz and Burris (1975) reported that N_2O was a noncompetitive inhibitor of C_2H_2 reduction. The source of the discrepancy with our earlier results is not clear. Perhaps there was an impurity in the N_2O used in the earlier experiments (NO is a very strong inhibitor of nitrogenase). In our current experiments we carefully purified N_2O gas by condensing it at a temperature below its melting point and then removing possible contaminating gases by evacuation. The fact that the current work utilized highly purified nitrogenase components, whereas the experiments of Rivera-Ortiz and Burris (1975) were performed on crude preparations with approximately 5% the specific activity, may have introduced some differences. We also tried to fit the experimental data to a noncompetitive program; the calculated K_{is} and K_{ii} were 0.143 ± 0.030 and −1.863 ± 0.423 atm, respectively. The K_{ii} was not defined for a reliable noncompetitive inhibition pattern.

The nonreciprocal response of N_2 or N_2O with C_2H_2 raises a puzzling problem. The competitive inhibition of C_2H_2 reduction by N_2 or N_2O suggests that these three substrates bind to the same form of the enzyme, whereas the noncompetitive inhibition of N_2 or N_2O reduction by C_2H_2 indicates that C_2H_2 can bind to an enzyme form that is distinct from the one to which N_2 or N_2O bind. Rivera-Ortiz and Burris (1975) proposed a model that involves the binding of C_2H_2 and N_2 at different sites and requires the enzyme to be at least six electrons reduced before it can start the reduction of N_2 to NH_3. They suggested that C_2H_2 reduction taps the same electron sink as N_2 does but can occur when the enzyme is two electrons reduced. This model predicts that the electron transfer from dinitrogenase to N_2 is in one step only; hence, there would be no observable intermediates. However, Thorneley et al. (1978) reported that an enzyme-bound dinitrogen–hydride intermediate was released upon acid or alkali hydrolysis of nitrogenase fixing N_2. This observation strongly suggests that N_2 is reduced step by step on the enzyme. It also was proposed that N_2 can bind to forms of dinitrogenase that are less than six electrons reduced (relative to the dithionite-reduced state) (Thorneley & Lowe, 1984). To explain the nonreciprocal relationship between N_2 or N_2O and C_2H_2, we suggest that C_2H_2 binds to two different redox forms of dinitrogenase, one of which also accommodates N_2 and N_2O. Infinitely high pC_2H_2 can completely eliminate the binding of N_2 or N_2O on the same enzyme form and therefore can restore C_2H_2 reduction to its maximal rate, but an infinitely high pN_2 or pN_2O cannot totally eliminate the other C_2H_2 binding form, and therefore the V_{max} for N_2 reduction cannot be achieved in the presence of C_2H_2. This model is supported by the observation made by Davis et al. (1979) that nitrogenase has two K_m's for C_2H_2. This indicates that there are two forms of the enzyme that bind C_2H_2 with different affinities. We observed the same phenomenon when testing the NO effect on C_2H_2 reduction (unpublished data). Unfortunately, our data do not define the K_m's of the two different C_2H_2 binding forms.

On the basis of the types of interactions among the substrates for nitrogenase, Hwang and Burris (1973) proposed that five sites or modified sites existed on nitrogenase. Shah et al. (1978) demonstrated that isolated iron–molybdenum cofactor from dinitrogenase of *A. vinelandii* reduced acetylene to ethylene with borohydride as an electron donor and suggested that FeMoco is an active site of nitrogenase and probably the site to which N_2 binds and where it subsequently is reduced. Several investigations have suggested that FeMoco is the only active site of nitrogenase; it was shown that C_2H_2 (Smith, 1983) and CH_3NC (Orme-Johnson, 1985) bind to the FeMoco center of the dinitrogenase. Hawkes et al. (1984) offered direct evidence that FeMoco is the site of N_2 binding and reduction. Some investigators have proposed that nitrogenase can exist in different redox forms and that different substrates are bound and reduced by these different enzyme forms (Chatt, 1980). As discussed earlier, N_2 and N_2O are mutually competitive and they have the same pattern of interaction with C_2H_2; therefore, we suggest that N_2 and N_2O bind to the same enzyme form, a conclusion at odds with the argument of Hwang et al. (1973) that N_2 and N_2O bind to different or modified sites on the enzyme. Evidence suggests that the determining factor for binding a certain substrate is the redox state of the enzyme. Upon passing two electrons to N_2O to produce N_2 and H_2O, the enzyme becomes less reduced; this form may be incapable of binding either N_2 or N_2O. Under such circumstances, an N_2 molecule just derived from N_2O would not be held at the active site. Because the dissociation of dinitrogenase reductase from dinitrogenase is the rate-limiting step of the nitrogenase reaction (Thorneley & Lowe, 1983), the N_2 molecule would have sufficient time to diffuse from the active site before dinitrogenase was reduced to a state such that it could bind N_2. In the interpretation of the results it also must be recognized that N_2O is a stronger inhibitor of N_2 reduction than is N_2 an inhibitor of N_2O reduction. In the usual measurements of N_2O reduction, the concentration of N_2O is much higher than that of N_2; therefore, the further reduction of N_2 to NH_3 is strongly inhibited by the substrate N_2O (Jensen & Burris, 1986).

In the absence of any other substrate, nitrogenase reduces protons from the aqueous medium to molecular H_2. All substrates inhibit H_2 evolution but to different extents. Although most of the substrates completely suppress H_2 evolution at high concentrations, it was predicted that the physiological substrate, N_2, would not block H_2 evolution completely even at an infinitely high pN_2 (Rivera-Ortiz & Burris, 1975); this prediction was tested experimentally and verified by Simpson

Scheme I



and Burris (1984). Nitrogenase under 50 atm of N_2 allocated approximately 27% of its electrons to H_2 production and 73% to the reduction of N_2 to NH_3. N_2O appears to bind to the same enzyme form as does N_2; however, a pN_2O of 40 atm completely abolishes H_2 production. When we included both N_2 and N_2O in the reaction system and measured the H_2 and NH_3 production, we observed that, at fixed pN_2, an increasing pN_2O decreased both NH_3 and H_2 formation. At fixed pN_2O, increasing the pN_2 increased the NH_3 production regardless of the pN_2O level. However, H_2 evolution declined with increasing pN_2 at lower pN_2O and increased with increasing pN_2 at higher pN_2O. The high-pressure experiment indicated that a high pN_2 increased both H_2 evolution and NH_3 production (see Figure 8). To explain the observed data, we propose (Scheme I) that H_2 is evolved from E_H^H via the back-reaction ($E_H^H \rightarrow E + H_2$) or by the displacement reaction when N_2 binds to E_H^H ($E_H^H + N_2 \rightarrow E^{N_2} + H_2$); N_2O also can bind to E_H^H and the two bound hydrides appear in H_2O ($E_H^H + N_2O \rightarrow N_2 + H_2O + E$). In the absence of N_2 and N_2O, nitrogenase will produce only H_2; when N_2 or N_2O is present, H_2 evolution will be partially suppressed, because a portion of the total electron flux will be diverted to N_2 or N_2O reduction. When N_2 or N_2O is present at infinitely high pressure, H_2 production by the back-reaction will be completely blocked. Therefore, there will be no H_2 produced under N_2O, but H_2 still will be produced via the displacement reaction under N_2, and the ratio of H_2 evolved to N_2 fixed will be 1:1. This model accommodates our experimental data: under a low pN_2O, H_2 evolution by the back-reaction was somewhat inhibited by N_2O, and an increase in the pN_2 caused further inhibition of this H_2-evolving pathway. Although some H_2 was produced concomitant with N_2 binding, the absolute gain of H_2 from this pathway was relatively small compared to the loss of H_2 caused by N_2 inhibition of the back-reaction, so N_2 induced a net decrease in H_2 evolution. Under a higher pN_2O, H_2 production via the back-reaction was strongly inhibited by N_2O. When the pN_2 was increased, the H_2 produced by the displacement reaction was noticeably higher than the additional inhibition that N_2 exerted on H_2 production via the back-reaction. Thus, a net increase in H_2 production was observed. The data do not rule out the possibility that N_2 merely releases the N_2O inhibition of H_2 evolution by enhancing the ability of the enzyme to produce H_2 from H^+.

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