*ACETYLENE AS A COMPETITIVE INHIBITOR OF N_2 FIXATION**

By R. Schöllhorn† and R. H. Burris

DEPARTMENT OF BIOCHEMISTRY, UNIVERSITY OF WISCONSIN, MADISON

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A variety of gases, including H_2, CO, N_2O, and NO, competitively inhibit N^2 fixation.[1] Schöllhorn and Burris[2] and Dilworth[3] independently observed that acetylene also inhibits N_2 fixation, and Schöllhorn and Burris[2] reported that the inhibition is competitive. Dilworth[3] observed that acetylene is reduced to ethylene by extracts from *Clostridium pasteurianum.*

Methods and Materials.—The gases H_2, N_2 (high purity), and acetylene (purified grade) were commercial cylinder gases. Acetylene was freed from traces of acetone by condensing the acetone in a trap cooled with dry ice.

Enzyme preparations: Cultures of *Clostridium pasteurianum* (strain W-5) were grown in a nitrogen-deficient medium with N_2, harvested, dried in a rotary evaporator, and stored in evacuated tubes at $-20°$. Extracts were obtained by autolyzing the dried cells for 1 hr with shaking in 0.05 *M* cacodylate buffer pH 6.8 at 32° in an atmosphere of H_2. The resulting suspension was centrifuged at 20,000 *g* for 25 min, and the supernatant was used.

Azotobacter vinelandii (strain 0) was grown in aerated liquid cultures in 180-liter glass-lined fermentors, and the cells were stored as a frozen paste. N_2-fixing extracts were prepared as described by Bulen, Burns, and LeComte[4] and the supernatant of successive centrifugations at 35,000 *g* for 30 min and 144,000 *g* for 60 min was used.

Experimental conditions: Experiments for inhibition of N_2 fixation in *C. pasteurianum* were run in 20-ml rubber-stoppered serum bottles containing 1 ml of reaction mixture and the desired atmosphere; H_2 was used as the electron donor. The mixture contained 5 μmoles ATP, 50 μmoles acetylphosphate, 2 μmoles $MgCl_2$, 50 μmoles cacodylate buffer at pH 6.8, and enzyme preparation containing about 8 mg protein. The bottles were shaken at 32° in a water bath, and the reaction was started by introducing the enzyme extract through a hypodermic needle. The reaction was stopped by addition of 1 ml of saturated K_2CO_3 solution, usually after 30 min. After microdiffusion to an acid-dipped glass rod inserted into the serum bottle, ammonia was determined spectrophotometrically by the Nessler method. Control reactions omitted H_2 or substituted an argon atmosphere. The average specific activity of the preparations for N_2 fixation was about 5 nanomoles N_2 fixed/mg protein × min at a pN_2 of 500 mm and a pH_2 of 200 mm.

Experiments for acetylene reduction were performed in 40-ml vessels with serum stoppers and 10 ml of reaction mixture, or in 100-ml vessels (containing 50 ml of reaction mixture stirred with a magnetic bar) equipped with a serum stopper and a three-way capillary stopcock. Reactions were stopped by introducing saturated ammonium sulfate solution. Ethylene formation was detected by mass spectrometry or gas chromatography. All quantitative ethylene determinations were performed with an Aerograph 700 gas chromatograph equipped with a 2-mm inside diameter aluminum column 160 cm long. The column was filled with activated alumina and was run at 145°. Helium served as carrier gas at flow rates of 0.5–2 cc/sec. The amounts of ethylene produced were determined by integration of the peaks obtained and comparison with a standard curve.

The reactions to examine the inhibition of preparations from *A. vinelandii* were run in 20-ml rubber-stoppered serum bottles containing 2 ml of reaction mixture and the desired atmosphere. Each bottle contained 0.2 mg creatine phosphokinase, 50 μmoles creatine phosphate, 5 μmoles ATP, 5 μmoles Mg^{++}, 80 μmoles cacodylate buffer pH 7.0, 40 μmoles $Na_2S_2O_4$, and about 9 mg of crude enzyme protein. Introduction of $Na_2S_2O_4$ and the enzyme started the reaction; the bottles were shaken for 40 min at 32°. The reactions were stopped with saturated K_2CO_3 solution, and the ammonia was determined as described for *C. pasteurianum.* Control reactions were run under argon.

Results.—Acetylene strongly inhibits fixation of N_2 by preparations from *C.*

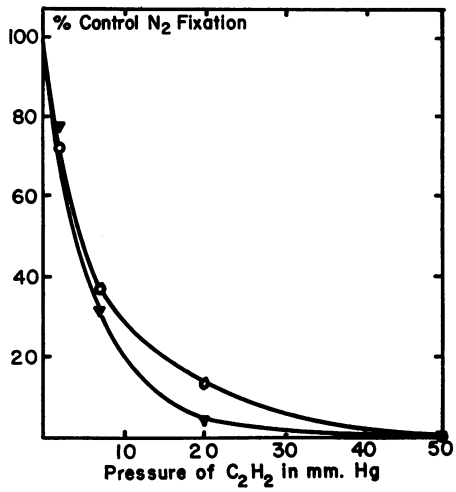

FIG. 1.—Inhibition by acetylene of N_2 fixation in extracts from *C. pasteurianum* and *A. vinelandii*. The 20-ml serum bottles contained the standard reaction mixtures described in the section on methods. O, *C. pasteurianum:* incubation time 30 min; atmosphere contained 400 mm N_2, 200 mm H_2, acetylene as indicated, and argon to 1 atm. ∇, *A. vinelandii:* incubation time 40 min; atmosphere contained 600 mm N_2, acetylene as indicated, and argon to 1 atm.

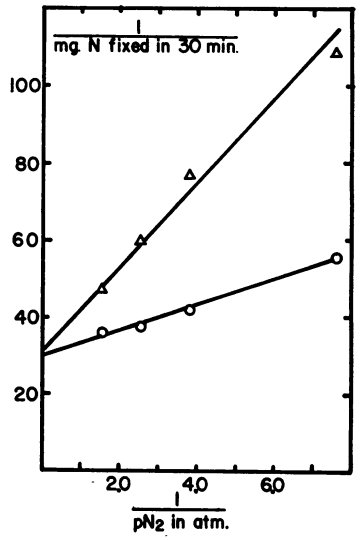

FIG. 2.—Inhibition of N_2 fixation in *C. pasteurianum* by acetylene. The system described in Fig. 1 was used. The H_2 pressure was 100 mm Hg and the incubation time was 30 min. Velocity was expressed as μg NH_3-N produced in 30 min. O, No acetylene; △, 2 mm Hg pressure of acetylene.

pasteurianum and from *A. vinelandii*. The inhibition was about 50 per cent at 4 mm partial pressure of acetylene and 400 or 600 mm partial pressure of N_2, respectively (Fig. 1). For both systems the dependence of inhibition on the pN_2 was determined at different partial pressures of acetylene. The reciprocal reaction velocity of N_2 reduction plotted against $1/pN_2$ for the acetylene pressures measured gave patterns characteristic of competitive inhibition with extracts from *C. pasteurianum* (Fig. 2) and *A. vinelandii* (Fig. 3).

Extracts from *C. pasteurianum* reduced acetylene to ethylene which was detected by mass spectrometry and gas chromatography; no ethane was found. The time course of acetylene reduction as measured by ethylene production was linear for at least 50 min (Fig. 4); the specific activity was 12 nanomoles acetylene reduced/mg protein × min at 200 mm partial pressure of acetylene.

Table 1 shows the increasing rate of acetylene reduction with an increasing acetylene partial pressure; a reciprocal plot of these data gives an estimated K_m value of 0.01 atm acetylene. Omission of components of the ATP generating system was accompanied by a decrease in the reduction of acetylene (Table 2).

Discussion.—Fixation of N_2 by enzyme preparations from *C. pasteurianum* or *A. vinelandii* is inhibited by comparable partial pressures of acetylene or carbon monoxide. Acetylene is isoelectronic and isosteric with N_2 and thus should fit easily into the N_2 chemisorbing site of the N_2-fixing enzyme.

The inhibition by acetylene was competitive with N_2 fixation in preparations from either *C. pasteurianum* or *A. vinelandii*. This competitive pattern was expected, as

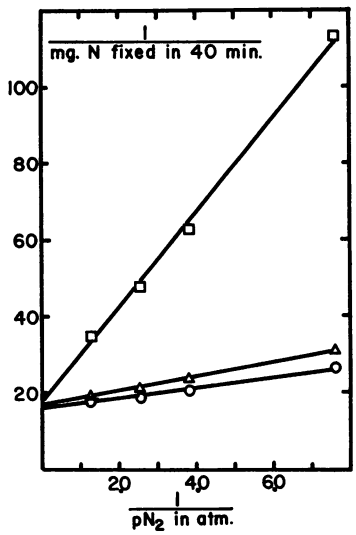

FIG. 3.—Inhibition of N_2 fixation in *A. vinelandii* by acetylene. The system described in Fig. 1 was used Velocity was expressed as μg NH_3-N produced in 40 min. O, No acetylene; △, 2 mm Hg pressure of acetylene; □, 5 mm Hg pressure of acetylene.

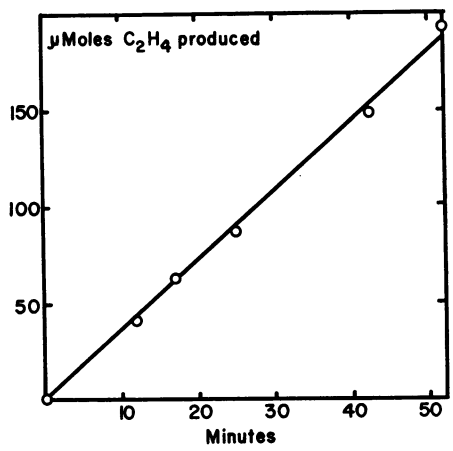

FIG. 4.—Time course of acetylene reduction by an extract from *C. pasteurianum*. Serum-stoppered 100-ml vessels contained 50 ml of standard reaction mixture. The pH_2 was 500 mm, the pC_2H_2 200 mm, and argon was added to 1 atm. C_2H_2 reduction was measured by C_2H_4 production.

experiments with *C. pasteurianum* showed that the acetylene molecule reacts with the enzyme and is reduced to ethylene as demonstrated by Dilworth.[3]

The specific activity of the enzyme for acetylene reduction was about 2.5 times greater than its activity for N_2 fixation; N_2 fixation involves a six-electron transfer to yield ammonia, whereas only two electrons are necessary for the single reduction step to convert acetylene to ethylene. The Michaelis constant of 0.01 atm (a value in agreement with that observed by Dilworth[3]) for acetylene reduction appears low compared to a K_m of about 0.1 atm for N_2 fixation. However, acetylene is much more soluble than N_2, and based upon the concentration of dissolved gases, the enzyme has somewhat greater affinity for N_2 than for acetylene. The acetylene reduction is dependent upon the presence of ATP and an electron donor such as H_2. Both the energy dependence and the specific competitive inhibition of N_2 fixation

TABLE 1

DEPENDENCE OF THE REACTION VELOCITY OF C_2H_2 REDUCTION UPON THE C_2H_2 PRESSURE

pC_2H (atm)	C_2H_4 produced (μmoles)
0.01	5.6
0.02	7.4
0.10	11.3

Reactions were performed in 40-ml vessels containing 10 ml of standard reaction mixture with the enzyme preparation carrying a total of 110 mg protein. Atmosphere: pH_2 600 mm, pC_2H_2 as indicated, argon to 1 atm. Incubation time: 5 min.

TABLE 2

ATP REQUIREMENT FOR ACETYLENE REDUCTION

System	Relative activity (%)
Complete	100
Minus H_2	0
" Mg^{++}	44
" acetyl phosphate	13
" Mg^{++}, acetyl phosphate ATP	0

Reactions were run in 40-ml bottles containing 10 ml of standard reaction mixture with 70 mg of protein from the enzyme preparation; components were omitted as indicated. The atmosphere consisted of 600 mm of H_2 and 100 mm of C_2H_2; the incubation time was 25 min.

suggest that the acetylene molecule is attached to and is reduced at the same enzyme site as the N_2 molecule. Whether the chemisorption of acetylene involves pure π bonds or opening of one π bond and formation of carbon-metal bonds, as in the case of heterogeneous catalytic hydrogenation, remains to be determined. It is possible that ethylene is not reduced, because its single π bond is not sufficient for chemisorption at the active site. Steric relationships also could be responsible for the observed lack of ethylene reduction, as ethylene has two hydrogen atoms more than the analog diimide which has been postulated at times to be the equivalent intermediate in N_2 fixation.

The establishment of the symmetrical hydrogenation of a triple bond in a molecule of high similarity to N_2 by the N_2-fixing enzyme complex and its reduction only to the level of ethylene constitute clear support for the hypothesis of N_2 fixation by stepwise reduction.

Summary.—Acetylene is a powerful and competitive inhibitor of N_2 fixation by preparations from *Clostridium pasteurianum* and *Azotobacter vinelandii*. The inhibition is about 50 per cent with 4 mm pressure of acetylene and 400 to 600 mm pressure of N_2. Extracts from *C. pasteurianum* reduce acetylene to ethylene if ATP and a suitable electron donor are supplied; the Michaelis constant for the reaction is about 0.01 atmosphere acetylene.

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† Fellow of the Deutscher Akademischer Austauschdienst. Present address: Department of Chemistry, Columbia University, New York City, N. Y.

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