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The Acetylene - Ethylene Assay for N_2 Fixation: Laboratory and Field Evaluation[1]

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Abstract. The methodology, characteristics and application of the sensitive C_2H_2–C_2H_4 assay for N_2 fixation by nitrogenase preparations and bacterial cultures in the laboratory and by legumes and free-living bacteria *in situ* is presented in this comprehensive report. This assay is based on the N_2ase-catalyzed reduction of C_2H_2 to C_2H_4, gas chromatographic isolation of C_2H_2 and C_2H_4, and quantitative measurement with a H_2-flame analyzer. As little as 1 $\mu\mu$mole C_2H_4 can be detected, providing a sensitivity 10^3-fold greater than is possible with ^{15}N analysis.

A simple, rapid and effective procedure utilizing syringe-type assay chambers is described for the analysis of C_2H_2-reducing activity in the field. Applications to field samples included an evaluation of N_2 fixation by commercially grown soybeans based on over 2000 analyses made during the course of the growing season. Assay values reflected the degree of nodulation of soybean plants and indicated a calculated seasonal N_2 fixation rate of 30 to 33 kg N_2 fixed per acre, in good agreement with literature estimates based on Kjeldahl analyses. The assay was successfully applied to measurements of N_2 fixation by other symbionts and by free living soil microorganisms, and was also used to assess the effects of light and temperature on the N_2 fixing activity of soybeans. The validity of measuring N_2 fixation in terms of C_2H_2 reduction was established through extensive comparisons of these activities using defined systems, including purified N_2ase preparations and pure cultures of N_2-fixing bacteria.

With this assay it now becomes possible and practicable to conduct comprehensive surveys of N_2 fixation, to make detailed comparisons among different N_2-fixing symbionts, and to rapidly evaluate the effects of cultural practices and environmental factors on N_2 fixation. The knowledge obtained through extensive application of this assay should provide the basis for efforts leading to the maximum agricultural exploitation of the N_2 fixation reaction.

To meet the imminent crisis in the world food supply (38) it is imperative that the resources of this planet be mobilized as rapidly and effectively as possible. Basic to such mobilization is a knowledge of the magnitude of the dynamic processes in the biosphere which affect the availability of nitrogen, the one element most often limiting in the production of foodstuffs (37). Of paramount importance in this context is the process of biological nitrogen fixation. Just as photosynthesis utilizes the freely available CO_2 of the atmosphere, nitrogen fixation draws on the unlimited supply of atmospheric nitrogen, and its potential role in increasing nitrogen availability has long been recognized. In spite of the importance of N_2 fixation very little accurate information is available to define the quantitative extent to which it occurs in the biosphere (35), and virtually nothing is known concerning the effects of various field practices on N_2 fixation. These gaps in our knowledge are attributable to the absence of effective methods for quantitative measurement of N_2 fixation *in situ*.

In the laboratory, N_2 fixation by living organisms has been measured by Kjeldahl analysis (7), ^{15}N-enrichment assayed by mass spectrometry (7), and ^{13}N-incorporation assayed by radioactive counting (8, 30); N_2 fixation by nitrogenase (N_2ase)[2] in cell-free extracts has been measured by ^{15}N-enrichment (9), ^{13}N-incorporation (8, 30), micro-Conway diffusion technique coupled with titrimetric (26) or colorimetric analysis of NH_3 (14), and N_2-H_2 uptake (27) or H_2 evolution (5) assayed manometrically. These methods are relatively insensitive except for the ^{13}N method, and its application is extremely limited because of its short half-life (10 minutes). Of these procedures only Kjeldahl analysis has been used to an appreciable extent for estimating N_2 fixation in field samples (35), but the method is insensitive and time-consuming. Although isotopic analysis of samples exposed to $^{15}N_2$ in the field has been used to demon-

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[2] The following abbreviations are used: N_2ase for nitrogenase; DTT for dithiothreitol; TES for N-tris (hydroxymethyl)-methyl-2-amino-ethanesulfonic acid; and MES for 2-(N-morpholino)ethanesulfonic acid; DEAE for diethylaminoethyl.

strate N_2 fixation *in situ*, the complexity and expense of this method have limited widespread application to field studies.

The opportunity for a novel approach to N_2 fixation analysis arose from demonstrations that N_2ase is a most versatile reducing catalyst (15, 20). The following reductions have been reported:

$N_2O \rightarrow N_2 + H_2O$ (17, 21a)
$N_3^- \rightarrow N_2 + NH_3$ (19, 31)
$C_2H_2 \rightarrow C_2H_4$ (13, 19, 23, 32)
$RCCH \rightarrow RCHCH_2$ (16a)
$HCN \rightarrow CH_4 + NH_3$ and tentatively CH_3NH_2 (16a, 19, 22a)
$RCN \rightarrow RCH_3 + NH_3$ (16a)
$RNC \rightarrow CH_4 + C_2H_4 + C_2H_6 + C_3H_8$ etc. (16a, 22a)

Schollhorn and Burris (31) and Dilworth (13) independently observed inhibition of N_2 fixation by C_2H_2 with extracts of *C. pasteurianum*: Schollhorn and Burris (32) established the competitive nature of this inhibition and Dilworth found C_2H_2 to be reduced to C_2H_4 in a reaction analogous to the reduction of N_2 to NH_3. The application of this reaction to a sensitive assay procedure for N_2-fixing activity was proposed by Hardy and Knight (19): "Utilization of the reduction of HCN to CH_4, of $H^{14}CN$ to $^{14}CH_3NH_2$, or of C_2H_2 to C_2H_4, and detection of CH_4 and C_2H_4 by hydrogen flame ionization after gas chromatography or detection of $^{14}CH_3NH_2$ may provide a sensitive new assay for detection of the N_2-fixing system. The gas chromatographic determination makes possible a range of about 10,000 times between minimum and maximum, in contrast to a 20-fold range with the NH_3 assay. C_2H_2 is the preferred assay substrate, since more product is formed because of its requirement for 2 electrons *versus* 6 electrons for HCN". Subsequently, Koch, Evans, and Russell (24, 25), Silver (33), Sloger and Silver (34) and Stewart, Fitzgerald, and Burris (36) have successfully employed C_2H_2 reduction coupled with C_2H_4 detection by H_2 flame ionization as an assay for N_2ase activity.

Since the original proposal by Hardy and Knight (19), the C_2H_2-C_2H_4 assay of N_2-fixing activity has undergone extensive development in this laboratory. This paper reports: 1) methodology of C_2H_2 and C_2H_4 analyses; 2) methodology of the C_2H_2-C_2H_4 assay of N_2-fixing activity *in situ*, and 3) characteristics of C_2H_2 reduction by N_2ase *in vitro* by cultures of N_2-fixing bacteria and by samples of the biosphere *in situ*. The results indicate that the C_2H_2-C_2H_4 assay of N_2-fixing activity is sensitive, universal, specific, rapid, simple, economical, and quantitative. Since this assay has the potential to promote revolutionary fundamental and practical advances, we believe that the C_2H_2-C_2H_4 assay of N_2 fixation represents one of the most important developments in N_2 fixation research. It is emphasized that the adoption of a consistent procedure by the various disciplines, *e.g.*, soil science, agronomy, marine biology, plant biology, microbiology, and biochemistry, which will utilize this method is essential if valid comparisons are to be made among results obtained from various sources.

Methods

Growth of Cells and Preparation of Extracts. Azotobacter vinelandii, ATCC 12518, was grown on nitrogen-free or urea media. Optical densities of bacterial cultures were determined in Will colorimeter tubes with a 650 mμ filter in a Lumetron colorimeter. Cells were broken and N_2ase was purified as previously described for N_2, N_2O, N_3^-, or HCN reduction experiments (5, 17, 18, 19). N_2ase was fractionated into the Mo-Fe protein fraction (also called Enzyme I) and Fe protein fraction (also called Enzyme II) (15) by published procedures (4, 22) modified to produce a discrete fractionation of the 2 components. Fractions were designated as follows: 1) crude extract—supernatant after 35,000 × *g* for 30 minutes; 2) heated extract—crude extract heated for 10 minutes at 60° under 0.5 atm of H_2 and centrifuged at 35,000 × *g* for 1 hour; 3) pre-protamine sulfate precipitate—phosphocellulose resolubilized precipitate between 0.0 to 0.1 mg protamine sulfate per mg protein of heated extract; 4) protamine sulfate precipitate—phosphocellulose resolubilized precipitate between 0.100 to 0.125 mg protamine sulfate per mg protein of heated extract; 5) protamine sulfate supernatant—supernatant after 0.125 mg protamine sulfate per mg protein of heated extract; 6) Mo-Fe protein fraction—fraction of protamine sulfate precipitate eluted from a DEAE-cellulose column in an anaerobic chamber by 0.20 M NaCl + 0.02 M $MgCl_2$ + 5 × 10^{-4} M DTT in 0.02 M tris·HCl at pH 7.0 following pre-elution with 0.15 M NaCl + 0.02 M $MgCl_2$ + 5 × 10^{-4} M DTT in 0.02 M tris·HCl at pH 7.0; and 7) Fe protein fraction—fraction eluted from above column by 0.35 M NaCl + 0.02 M $MgCl_2$ + 5 × 10^{-4} M DTT in 0.02 M tris·HCl at pH 7.0; All fractions were stored anaerobically with fractions 4 and 7 stored at room temperature. Fraction 6 contained Fe and Mo and fraction 7 contained Fe as reported by others (4). Neither fraction was homogeneous by gel electrophoresis.

Clostridium pasteurianum, ATCC 6013, was grown on nitrogen-free or NH_4Cl media (9). Dried cells were broken by autolysis as previously described for N_2, N_2O, N_3^-, or HCN reduction experiments (9, 17, 19). An extremely sharp fractionation of N_2ase into its 2 components was produced by modification of an old procedure (29). Fractions were designated as follows: 1) crude extract—supernatant after 35,000 × *g* for 30 minutes of autolysate; 2) negative phosphate gel preparation—supernatant from crude extract treated with protamine sulfate to remove nucleic acids and with calcium phosphate gel to remove inactive protein (29); 3) Mo-Fe protein fraction—fraction eluted from DEAE-cellulose

between 0.1 to 0.3 M NaCl in 0.01 M KPO_4, pH 7.0, after 15 minutes anaerobic batch treatment of a phosphate gel preparation with 8 mg damp DEAE-cellulose per mg protein. This fraction contains Mo and Fe and is stable for months when stored anaerobically at 4°. In some cases it was further purified by anaerobic chromatography on DEAE-cellulose with elution by 0.29 M NaCl in 0.01 M tris·HCl, pH 7.0. This fraction is not homogeneous by gel electrophoresis. 4) Fe-protein fraction—crude extract heated for 10 minutes at 60° under 0.5 atm H_2 and centrifuged at 35,000 × *g* for 30 minutes; this fraction was stored under anaerobic conditions at room temperature. Fraction 3 or 4 could only be obtained from N_2-grown cells.

Clostridium butyricum, Lactobacillus leichmanii, Bacillus subtilis, Serratia marcescens, Escherichia coli, Streptococcus lactis, Saccharomyces cerevisiae, Bacillus cereus var. *mycoides, Pseudomonas aeruginosa, Pseudomonas fluorescens, Aerobacter aerogenes, Staphylococcus epidermidis, Sarcina lutea, Spirillum itersonii, Proteus vulgaris, Alcaligines faecalis*, and *Rhodospirillum rubrum* were the kind gifts of Dr. R. Bailey of the University of Delaware and were grown on appropriate media containing fixed nitrogen. *Rhizobium japonicum*, ATCC 10324, *R. meliloti*, ATCC 10312, *R. leguminosarum*, ATCC 10004, *Rhizobium sp.*, ATCC 10317, and *R. trifolii*, ATCC 10328, were grown on a medium containing in g per liter: K_2HPO_4, 1.0; KH_2PO_4, 1.0; $MgSO_4 \cdot 7H_2O$, 0.36; $CaSO_4 \cdot 2H_2O$, 0.17; $FeCl_3 \cdot 6H_2O$, 0.005; KNO_3, 0.7; yeast extract, 1.0; and mannitol, 3.0 (10).

Growth of Legumes. Field-grown soybeans (*Glycine max* Merr. var. Wayne) were sown on May 15, 1967, in 38-inch rows by a commercial grower in Chester County, Pennsylvania. Standard agricultural practices including seed inoculation with commercial inoculum and recommended additions of phosphorus and potassium but no nitrogen were used. In addition, soybeans and other legumes (*Phaseolus vulgaris, Medicago sativa, Arachis hypogea*, and *Pisum sativum*) were grown in sterilized Perlite using a nitrogen-free nutrient solution (1) in a greenhouse or in controlled environment growth chambers. A normal day-night regime of 16 hours, 24°, and 8 hours, 18° was maintained in Sherer-Gillett Model CEL255-6 chambers operated at maximum light intensity during the light period. Other samples of legumes and soils collected within a radius of 200 miles of Wilmington include samples from the Jordan Fertility Plots through the courtesy of Professors A. Richer and E. S. Lindstrom of Pennsylvania State University, and from the Georgetown Experimental Station through the courtesy of Professor R. Cole of the University of Delaware. Indicated times are Eastern Daylight Saving Time. The date of bud opening was recorded as the date of flowering, and the first indication of leaf yellowing was recorded as the onset of senescence.

Assays. Reductions of N_2 or C_2H_2 by N_2ase preparations or cultures of bacteria were performed in 40 ml incubation vessels sealed with serum caps. For N_2ase preparations dithionite was dissolved in O_2-free water containing a pre-determined quantity of acid or base to produce a final pH of 7. The energy source and reductant were placed in the sidearm, the extract and other components were placed in the main compartment, and the incubation flask was immediately evacuated. After repeated flushing with the indicated gas, the contents of the sidearm were tipped in to initiate the reaction. For cultures of bacteria, the incubation was initiated by the aseptic addition of the bacteria to the sealed incubation vessel containing appropriate medium and gas phase. The reaction mixture or culture was incubated on a rotary shaker at 30° for the indicated time, and the incubation was stopped by the addition of 0.5 ml of 6 N H_2SO_4. Samples of gas phase were analyzed with a mass spectrometer utilizing the initial gas phase as an internal standard or with a H_2-flame ionization detector after gas chromatographic separation (see below). Nitrogen fixation by N_2ase in extracts was measured by titration of NH_3 after micro-diffusion (26), and N_2 fixation by cultures was measured by Kjeldahl analysis of 5 ml aliquots. Deuterated ethylenes were analyzed in a Perkin-Elmer Model 21 infrared spectrophotometer using a 3.3 cm micro gas cell.

Assay of Acetylene and Ethylene. In early work an activated alumina column at 150° and a Perkin-Elmer 880 or 800 gas chromatograph with a dual H_2-flame ionization detector were used (19). Subsequently, a one-eighth inch × 10 foot column containing 20 % ethyl, N′,N′-dimethyl oxalamide on 100 to 120 mesh acid-washed firebrick at 0° with a He flow rate of 30 ml/minute has been found to be most effective for gas chromatographic separation of acetylene and ethylene as well as other saturated and unsaturated hydrocarbons containing up to 4 carbons. Modified Perkin-Elmer F-11 gas chromatographs equipped with H_2-flame ionization detectors are utilized. Representative retention times in minutes are: methane, 0.8; ethane, 1.0; ethylene, 1.1; propane, 1.4; propylene, 1.9; isobutane, 2.1; butane, 2.8; acetylene, 3.8; 1-butene, 4.4; isobutylene, 4.5; allene, 4.8; trans-2-butene, 5.2; cis-2-butene, 6.2; methylacetylene, 10.4. A typical chromatogram of a standard mixture of C_2H_2 (0.1 atmosphere) and C_2H_4 (2.5×10^{-4} atmosphere) is shown in figure 1a, and a chromatogram of C_2H_2 (0.1 atmosphere initial pressure) and C_2H_4 produced by a culture of N_2-grown *Clostridium pasteurianum* in figure 1b. The symmetry of C_2H_2 and C_2H_4 peaks and the absence of other components are indicated in figure 1b. A standard curve of peak height *vs.* C_2H_4 or C_2H_2 content of injected sample (fig 2) demonstrates the linear response and sensitivity of the assay. Less than 10^{-12} moles of C_2H_4 can be detected per injected sample of 200 μl. Ethylene content can be calculated from this standard curve, or

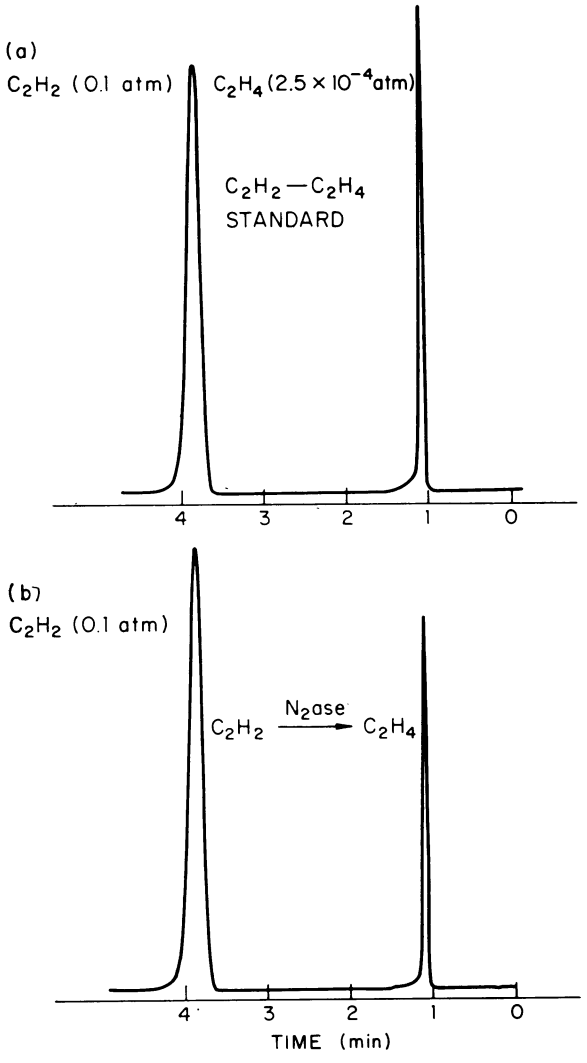

FIG. 1. Typical chromatograms of a) a known mixture of 200 μl of C_2H_2 (0.1 atm) C_2H_4 (2.5×10^{-4} atm) and He to 1 atm, and b) 200 μl of the gas phase of an incubation after N_2ase-catalyzed reduction of 0.1 atm C_2H_2. An ester-amide gas chromatographic column was used and detection was by hydrogen flame ionization (see under Methods).

alternatively the "built-in" internal standard, C_2H_2, can be used since both C_2H_2 and C_2H_4 are determined. Our broad experience with the C_2H_2–C_2H_4 assay (over 2000 samples assayed) indicates that C_2H_2 is a valid and useful internal standard, since with the exception of large nodulated plant roots less than 2 % of the initial C_2H_2 (0.1 atmosphere) is converted to C_2H_4 during a 1 hour incubation.

Reagents. ATP, GTP, CTP, UTP, creatine phosphate, creatine kinase (ATP:creatine phosphotransferase, EC 2.7.1.40), and protamine sulfate were obtained from Sigma Chemical Company; $Na_2S_2O_4$, reagent grade, from Fisher Scientific Company; He, A, N_2, CO, and C_2H_2 as highest purity available

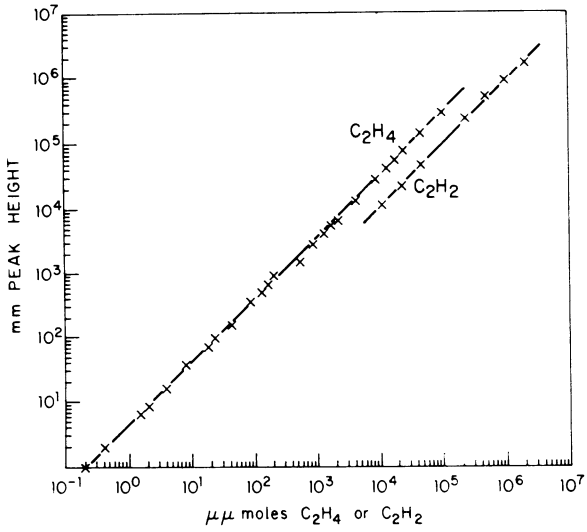

FIG. 2. A standard curve of peak heights of C_2H_2 and C_2H_4 determined with the gas chromatographic system of figure 1.

from The Matheson Company. Acetone was removed from C_2H_2 by a concentrated H_2SO_4-scrubber and corrections were made for the C_2H_4 content of C_2H_2. The C_2H_4 impurity in C_2H_2 from a given cylinder must be determined daily since it varies inversely with the pressure in the cylinder.

Results

C_2H_2 Reduction by N_2ase in vitro. Reduction of C_2H_2 to C_2H_4 by N_2ase of cell-free extracts of *A. vinelandii* was examined with respect to a wide variety of characteristics, and the striking similarities between N_2 fixation and C_2H_2 reduction are reported in this section.

Requirements and Products of C_2H_2 Reduction. Reduction of C_2H_2 to C_2H_4, like reduction of N_2 to 2 NH_3 (3, 12, 16, 25, 27), requires an enzyme extract containing N_2ase, an energy source, and a reductant (table I). A similar energy and reductant requirement has been reported for reduction of acetylene by extracts of *C. pasteurianum* and soybean bacteroids (13, 25, 29a, 32). Extracts of urea-grown cells do not have N_2ase activity and do not have C_2H_2-reducing activity. No C_2H_4 formation is found in the absence of C_2H_2. Detectable amounts of C_2H_6 or CH_4 are not formed by the complete system capable of reducing C_2H_2 (sensitive analyses indicate that C_2H_6 can be no more than 0.01 % as abundant as C_2H_4); furthermore, ethylene is not reduced to C_2H_6 or CH_4 by *Azotobacter* N_2ase in a complete system. Thus, the N_2ase-catalyzed reaction appears to be quite specific for the reduction of C_2H_2 only to C_2H_4.

Specific Requirement for ATP. The sensitivity of the C_2H_2–C_2H_4 assay of N_2ase permits a deter-

Table I. *Requirements and Product of C_2H_2 Reduction by* Azotobacter *N_2ase*

Complete system contained per ml in μmoles: tris•HCl, 50; creatine phosphate (CrP), 56; ATP, 5; $Na_2S_2O_4$, 20 (all at pH 7.0); and $MgCl_2$, 5; and in mg proteins: heated extract of N_2-grown or NH_3-grown *A. vinelandii*, as indicated, 4; and creatine kinase (CrK), 0.2. Gas volume 36 ml; liquid volume, 4 ml; incubation time, 30 min; temperature, 30°. Aliquots of gas phase assayed by gas chromatography on alumina column.

Incubation system	Gas phase	C_2H_4	C_2H_6	CH_4
		μmoles/incubation		
Requirements complete, N_2-grown	0.05 atm C_2H_2 0.95 atm He	24	<0.001	<0.002
Minus enzyme or CRP, CrK, ATP or $Na_2S_2O_4$	0.05 atm C_2H_2 0.95 atm He	<0.001	<0.001	<0.002
Minus C_2H_2	1.0 atm He	<0.001	<0.001	<0.002
Specificity for N_2-grown cells				
complete, N_2-grown	0.1 atm C_2H_2	20.2	<0.001	<0.002
complete, NH_3-grown	0.1 atm C_2H_2	<0.001	<0.001	<0.002
Stability of C_2H_4 complete, N_2-grown	0.1 atm C_2H_4 0.9 atm He	150	<0.001	<0.002

Table II. *Specificity of Phosphagen Requirement for C_2H_2 Reduction by* Azotobacter *N_2ase*

Complete incubation system, table I, except replacement of creatine phosphate, and creatine kinase by 10 mM ATP, GTP, CTP, or UTP, pH 7.0; 2.2 mg protein of a protamine ppt of N_2ase of *A. vinelandii*. Liquid volume, 2 ml; gas volume, 38 ml; incubation time, 5 min; gas phase, 0.1 atm C_2H_2, 0.9 atm He. Aliquots of gas phase assayed by gas chromatography on ester-amide column.

Phosphagen	mμmoles C_2H_4/incubation
ATP	1440
CTP	0.8
GTP	<0.5
UTP	<0.5

mination of the specificity of its requirement for nucleoside triphosphate. An *Azotobacter* N_2ase preparation was incubated with C_2H_2, reductant and ATP, UTP, CTP, or GTP as the sole energy source (table II). Only ATP supported C_2H_2 reduction indicating that the energy requirement of N_2ase is very specific for ATP. Burns has observed similar phosphagen specificity for the energy-dependent H_2-evolution activity of N_2ase (15).

Enzyme Level. The rate of C_2H_2 reduction is related to N_2ase concentration in a sigmoidal fashion (fig 3a); the plot of the rate of N_2 fixation *vs.* enzyme concentration does not extrapolate linearly to zero enzyme (3, 19) and might show a sigmoidal relationship if the NH_3 assay were sufficiently sensitive to measure NH_3 formation at the lower limits of enzyme concentration. The plot of the rate of C_2H_2 to C_2H_4 reduction *vs.* enzyme is consistent with a 2-component system:

Mo-Fe protein + Fe protein ⇌ N_2ase

Time Course. The rate of C_2H_2 reduction *vs.* time is linear for about 45 minutes, and the reaction

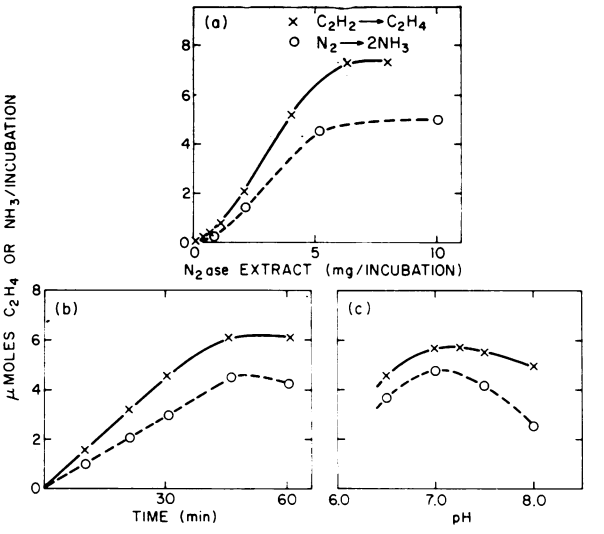

FIG. 3. Reduction by *Azotobacter* N_2ase of $C_2H_2 \rightarrow C_2H_4$ and $N_2 \rightarrow 2NH_3$ *versus* a) enzyme level, b) time, and c) pH. Complete incubation system, table I; liquid volume, 2 ml; gas volume, 38 ml; time, 30 min unless otherwise indicated; gas phase, 0.1 atm C_2H_2 + 0.9 atm He for $C_2H_2 \rightarrow C_2H_4$ and 1 atm of N_2 with 1 atm He as control for $N_2 \rightarrow 2NH_3$. Mixture of TES and MES used to vary pH from 6.5 to 8.0.

stops when ATP is exhausted, in complete analogy to the N_2 reduction reaction (fig 3b).

pH Maximum. Nitrogenase-catalyzed C_2H_2 reduction was examined from pH 6.5 to pH 8.0 (fig 3c). Maximum activity occurred over a wide range near pH 7.0, which is similar to that observed for N_2 (3) or other reducible substrates of N_2ase (21).

Km of C_2H_2. Nitrogenase is saturated by 0.03 to 0.10 atmosphere C_2H_2 (fig 4a) while 0.5 atmosphere has been observed to inhibit C_2H_2 reduction

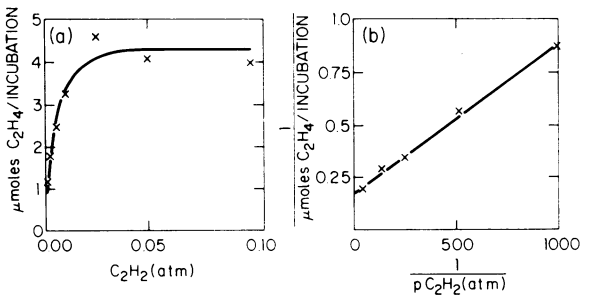

FIG. 4. a) Ethylene formation from C_2H_2 as a function of pC_2H_2 by *Azotobacter* N_2ase, and b) plot of reciprocal velocity *versus* reciprocal pC_2H_2 for determination of K*m* of C_2H_2. Complete incubation system, table I; liquid volume, 2 ml; gas volume, 38 ml; time, 30 min; gas phase, indicated pC_2H_2 plus He to 1 atm.

and reductant-dependent ATPase activity of N_2ase. This inhibition at 0.5 atmosphere C_2H_2 may be due to C_2H_2 or to possible trace impurities in C_2H_2. A typical plot of reciprocal velocity of C_2H_2 reduction *vs.* reciprocal pC_2H_2 is shown in figure 4b. Estimated Michaelis constants of 0.002 to 0.009 atmosphere of C_2H_2, with an average value of 0.004 atmosphere, have been obtained. A tentative K*m* of 0.01 atm has been reported for clostridial N_2ase (13). Michaelis constants of 0.05 to 0.17 atmosphere of N_2 have been reported for N_2ase (14, 19, 25, 27). Based on partial pressures, the estimated K*m* of C_2H_2 is only about 5 % that of N_2. Based on the calculated concentrations of C_2H_2 and N_2 in an aqueous solution, the estimated K*m* of C_2H_2, 0.1 to 0.3 mM, is similar to that of N_2, 0.03 to 0.1 mM.

Table III. *Inhibition of ATP-Dependent H_2 Evolution by N_2 and C_2H_2*

Complete system, table I; liquid volume 2 ml; gas volume 38 ml; N_2ase preparation, heated extract of *A. vinelandii*, 7.5 mg; gas phase, as indicated plus He to 1 atm. Hydrogen determined by mass spectrometric analysis of gas phase with He as an internal standard.

Added substrate	H_2	ΔH_2	% Inhibition of H_2 evolution
	mμmoles per min per mg protein		
None	71	0	0
N_2, 0.5 atm	18	53	75
C_2H_2, 0.1 atm	11	61	85

Inhibition of H_2 Evolution. The ATP-dependent H_2-evolving activity of N_2ase is decreased by N_2, N_3^-, or N_2O reduction, and the decrease in H_2 evolved is equivalent in electrons to those required for reduction of N_2 to 2 NH_3, N_3^- to N_2 + NH_3, or N_2O to N_2 + H_2O (4, 17, 19, 25). Reduction of C_2H_2 to C_2H_4 also inhibits H_2 evolution by clostridial (13, 32) and *Azotobacter* N_2ase (21). Inhibition by a saturating level of C_2H_2 may be greater than by that of N_2, *e.g.*, 85 % for C_2H_2 and 75 % for N_2 (table III).

Stoichiometry of C_2H_2 Reduction. An excellent balance exists between the concomitant decrease in C_2H_2, increase in C_2H_4, and decrease in H_2 evolution (table IV) during N_2ase-catalyzed reduction of C_2H_2. Thus, reduction of C_2H_2 decreased C_2H_2 by 13.8 μmoles and increased C_2H_4 by 14.5 μmoles, supporting the following relationship:

$$nC_2H_2 \xrightarrow{N_2\text{ase}} nC_2H_4$$

The decrease of 12.3 μmoles in H_2 evolution (equivalent to 24.6 μmoles of electrons) produced by C_2H_2 reduction corresponds to the formation of 14.5 μmoles of C_2H_4 (equivalent to 29 μmoles of electrons) and indicates the following electron balance:

(H_2 evolved)$_{-C_2H_2}$ = (H_2 evolved + C_2H_4 formed)$_{+C_2H_2}$

Addition of 0.18 atmosphere CO inhibited C_2H_2 reduction and restored H_2 evolution. Since the loss of C_2H_2 can be accounted for as C_2H_4 and the loss of electrons evolved as H_2 can be accounted for as the electrons required for C_2H_2 reduction, no significant product of C_2H_2 reduction in addition to C_2H_4 is indicated. Furthermore, the equivalence between the decrease in electrons evolved as H_2 and

Table IV. *Stoichiometry of C_2H_2 Reduction by* Azotobacter *N_2ase*

Complete system, table I; liquid volume, 4 ml; gas volume, 36 ml; incubation time, 30 min; aliquots of gas phase assayed by mass spectrometry.

Incubation system	Gas phase	C_2H_2	C_2H_4	C_2H_6	CH_4	H_2
		μmoles/incubation				
Complete	0.02 atm C_2H_2 0.18 atm He	15.5	14.5	0.0	0.0	11.0
Minus enzyme or CrP, CrK, ATP, or $Na_2S_2O_4$	0.02 atm C_2H_2 0.18 atm He	29.3	0.0	0.0	0.0	0.0
Minus C_2H_2	0.2 atm He	0.0	0.0	0.0	0.0	23.3
Complete	0.02 atm C_2H_2 0.18 atm CO	25.0	0.0	0.0	0.0	24.7

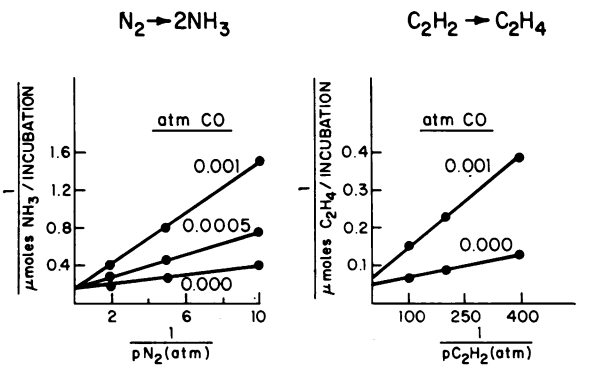

FIG. 5. Competitive inhibition of $C_2H_2 \rightarrow C_2H_4$ and $N_2 \rightarrow 2NH_3$ by CO using *Azotobacter* N_2ase. Complete incubation system, table I; liquid volume, 2 ml; gas volume, 38 ml; time, 30 min; gas phase, indicated pC_2H_2 or pN_2 plus indicated pCO and He or A to 1 atm. N_2 is replaced by A as control for $N_2 \rightarrow 2NH_3$.

the electrons used for ethylene formation indicates that at least the proposed electron-activating site of N_2ase (15, 20) is involved in C_2H_2 reduction, as has been proposed for N_2O and N_3^- reductions (17, 19, 21a).

Inhibition of C_2H_2 Reduction by CO. Carbon monoxide is a competitive inhibitor of N_2 fixation (15, 20, 25a). Figure 5 demonstrates that CO is also a competitive inhibitor of C_2H_2 reduction by *Azotobacter* N_2ase. Furthermore, the similar CO inhibition constants of 2.9×10^{-4} and 3.1×10^{-4} atmosphere for N_2 fixation and C_2H_2 reduction, respectively, provide indirect support that the substrate-complexing site of N_2ase (15, 20) is involved in the reduction of C_2H_2 as well as N_2.

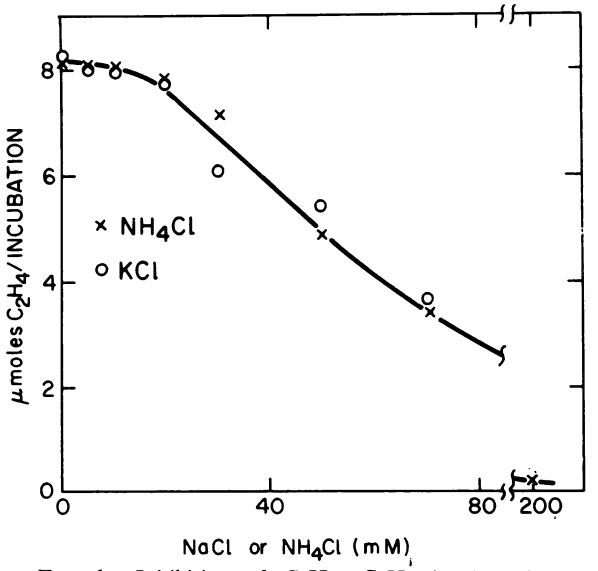

FIG. 6. Inhibition of $C_2H_2 \rightarrow C_2H_4$ by NH_4Cl or NaCl. Complete incubation system with *Azotobacter* N_2ase, table I; liquid volume, 2 ml; gas volume, 38 ml; time, 30 min; gas phase, 0.1 atm C_2H_2, 0.9 atm He; NH_4Cl or KCl as indicated.

Effect of NH_4^+ and Na^+ on $C_2H_2 \rightarrow C_2H_4$ Reduction. Ammonia is the product of N_2 fixation; however, N_2 fixation appears to be relatively insensitive to added NH_4^+ (9). This insensitivity suggests that the product of N_2 fixation does not effectively compete with N_2 for the substrate-complexing site of N_2ase and that NH_4^+ does not control activities of N_2ase associated with electron-activation. Reduction of C_2H_2 to C_2H_4 provides an opportunity to determine if there is a specific effect of NH_4^+ on other N_2ase-catalyzed reductions. Figure 6 indicates no specific inhibition by NH_4^+, since C_2H_2 reduction is equally sensitive to NH_4^+ or Na^+ with 50 % inhibition produced by 50 to 70 mM NH_4Cl or NaCl.

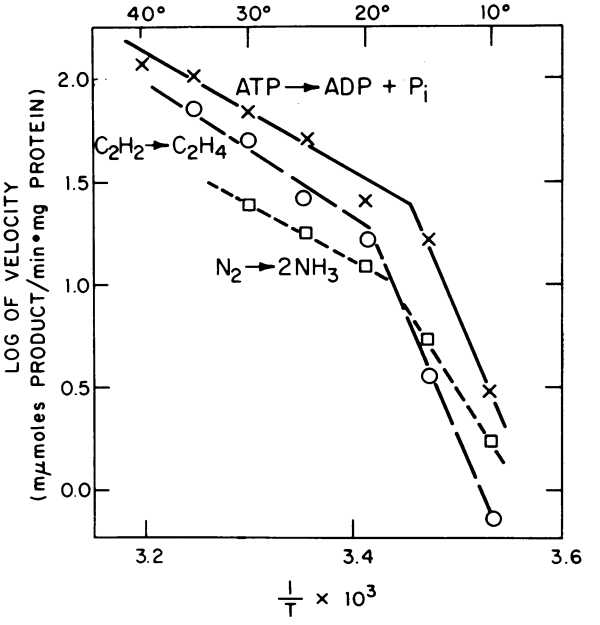

FIG. 7. Arrhenius plots of $C_2H_2 \rightarrow C_2H_4$, $N_2 \rightarrow 2NH_3$ and ATP→ADP + P_i by *Azotobacter* N_2ase in the range 10° to 40°. Complete incubation system, table I; liquid volume, 2 ml; gas volume, 38 ml; time, 30 min for C_2H_2 or N_2 reduction, 15 min for ATP hydrolysis; gas phase, 0.1 atm C_2H_2 plus He to 1 atm for $C_2H_2 \rightarrow C_2H_4$, 1 atm N_2 with 1 atm He as control for $N_2 \rightarrow 2NH_3$, and 1 atm He for ATP→ADP + P_i. Points represent averages of 3 samples.

Activation Energy of C_2H_2 Reduction. The activation energies for reduction of N_2 and other reactions of N_2ase, including ATP-dependent H_2 evolution and reductant-dependent ATPase, have been recently determined (6, 21). A break in the Arrhenius plots for all these activities is observed near 20° with similar but lower activation energies above (13–15 kcal/mole) and similar but higher activation energies below this point (35–50 kcal/mole). Arrhenius plots of C_2H_2 reduction also show a similar break and similar activation energy (fig 7).

Stereochemistry of C_2H_2 Reduction. Ethylene formed from C_2H_2 by reduction by *Azotobacter* N_2ase in a 99.8% D_2O system was examined by infrared spectrophotometry in order to identify the deuterated species (fig 8). cis-1,2-Dideuteroethylene (843 cm^{-1}) is the major product, as reported for clostridial N_2ase (13). A small amount of monodeuteroethylene (1000 cm^{-1}) and a possible trace of trans-1,2-dideuteroethylene (988 cm^{-1}) were found. These results indicate that neither of the original hydrogens of acetylene is replaced during reduction and that acetylene may be complexed to the substrate-complexing site of N_2ase *via* a "side-on" orientation.

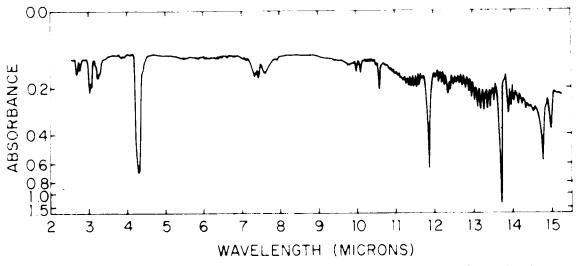

FIG. 8. Infrared spectrum of deuterated ethylenes produced by reduction of C_2H_2 by *Azotobacter* N_2ase. Complete incubation system, table I; liquid volume, 10 ml; gas volume, 30 ml; time, 30 min; gas phase 0.1 atm C_2H_2; all reagents in 99.8% D_2O and protamine sulfate ppt of N_2ase resuspended in 99.8% D_2O.

Fractionation of N_2ase and N_2-Fixing and C_2H_2-Reducing Activities. Nitrogen-fixing extracts of *A. vinelandii* were fractionated according to the established procedure in this laboratory for N_2ase purification. The N_2- and C_2H_2-reducing activities paralleled each other, and the ratio of C_2H_4 formed to N_2 fixed was found to be in the range of 3 to 4.5 (table V).

Recombination of Mo-Fe Protein and Fe Protein Fractions of N_2ase. Nitrogenase can be separated into 2 protein fractions (4, 22). One contains Fe and Mo and is called the Mo-Fe protein fraction; the other contains Fe and is called the Fe protein fraction. Neither individual protein fraction has biological activity, but N_2ase, the complex formed by the protein fractions, is active for N_2 reduction.

Table V. *C_2H_2 and N_2 Reduction by* Azotobacter *N_2ase Preparations*

Complete incubation system, table I; liquid volume, 2 ml; gas volume, 38 ml; N_2ase preparation, as indicated; gas phase, 0.1 atm C_2H_2, 0.9 atm He for C_2H_2 reduction, 1 atm N_2 with 1 atm He as control for N_2 fixation. C_2H_4 assayed gas chromatographically; NH_3 assayed titrimetrically.

N_2ase preparation	Protein mg/incubation	$C_2H_2 \rightarrow C_2H_4$ μmoles/incubation	$N_2 \rightarrow 2NH_3$ μmoles/incubation	$\frac{C_2H_2 \rightarrow C_2H_4}{N_2 \rightarrow 2NH_3}$
Heated extract	4.3	5.00	1.35	3.7
Pre-protamine precipitate	4.0	<0.002	0.00	
Protamine precipitate	0.91	6.02	1.43	4.2
Protamine supernatant	3.6	<0.002	0.00	

Table VI. *C_2H_2 Reduction by Mo-Fe Protein and Fe Protein Fractions of* Azotobacter *and Clostridial N_2ase*

Complete incubation system, table I; liquid volume, 2 ml; gas volume, 38 ml; incubation time, 30 min; gas phase, 0.1 atm C_2H_2, 0.9 atm He; protein fraction as indicated (see under Methods for preparation and designation of fractions). Data from 2 experiments are shown. C_2H_4 assayed gas chromatographically on ester-amide column.

Fraction	Organism	μmoles C_2H_4/incubation		mg Protein/ incubation
Crude extract	*Clostridium*	1.38		8.7
Mo-Fe protein	"	0.0007	0.0003	1.5
Fe-protein	"	0.0073	0.0003	5.0–8.5
Mo-Fe protein	"	1.72	1.10	1.5
+ Fe protein	"			5.0
Protamine ppt.	*Azotobacter*	7.10		4.2
Mo-Fe protein	"	0.152	0.0008	2.4–4.5
Fe protein	"	0.032	0.0214	2.2–2.9
Mo-Fe protein	"	4.55	2.73	2.4–4.5
+ Fe protein	"			2.2–2.9
Mo-Fe protein	*Azotobacter*	...	0.006	2.4
+ Fe protein	*Clostridium*			8.5
Mo-Fe protein	*Clostridium*	0.102	...	1.5
+ Fe protein	*Azotobacter*			2.9

ATP-dependent H_2 evolution, and reductant-dependent ATPase (4, 22). Nitrogenases of *C. pasteurianum* and *A. vinelandii* were separated into their Mo-Fe protein and Fe protein fractions as described under Methods. Acetylene reduction was determined with the individual fractions, the recombination of the individual fractions from the same species and the cross-combination of the proteins from different species (table VI). No nitrogen-fixing activity remained in the individual fractions, but it was restored by recombination of the fractions. Neither the Mo-Fe protein fraction nor the Fe protein fraction has appreciable C_2H_2-reducing activity, and thus represent the "lowest activity" fractions that have been reported, *e.g.*, our best preparations of Mo-Fe protein (*Clostridium*), Fe protein (*Clostridium*), Mo-Fe protein (*Azotobacter*), and Fe protein (*Azotobacter*) have less than 0.04, 0.02, 0.03, and 0.7 %, respectively, of their recombined activities. Recombination of the 2 protein fractions of *Azotobacter* produces stimulations up to 123-fold, and of *Clostridium* up to 1080-fold. A recent report indicates an enhancement of activity of 5.5-fold by recombination of *Azotobacter* fractions (22). Cross-combination of Mo-Fe protein (*Azotobacter*) + Fe protein (*Clostridium*) or Mo-Fe protein (*Clostridium*) + Fe protein (*Azotobacter*) produces $<\sim 5$ % of the C_2H_2-reducing activity found in the recombination within species experiment. The above cross-combinations have been reported to produce no N_2-fixing activity (11).

These experiments were designed to demonstrate the absence of C_2H_2-reducing activity in each fraction and the presence of this activity in recombined fractions from the same species. Specific activities were not maximized by the addition of an excess of one fraction to a limiting amount of the fraction whose activity is to be maximized.

C_2H_2 Reduction by Bacterial Cells. Characteristics of the reduction of C_2H_2 to C_2H_4 by N_2-fixing cultures of *A. vinelandii* and *C. pasteurianum* are reported in this section. These results indicate the validity of the C_2H_2–C_2H_4 assay of N_2 fixation with living organisms and complement results reported in the previous section with N_2ase preparations from *Azotobacter* and *Clostridium*.

Requirements and Products of C_2H_2 Reduction. Acetylene is reduced to C_2H_4 by N_2-grown cells of *A. vinelandii* and *C. pasteurianum* (table VII). Extracts from these cells contain N_2ase and reduce C_2H_2 to C_2H_4. Control cultures grown on fixed nitrogen sources reduced less than 0.1 % (*Azotobacter*) and less than 5 % (*Clostridium*) the amount of C_2H_2 reduced by the N_2-grown cultures. Extracts from these cells contain little or no N_2ase and do not reduce C_2H_2. Neither ethane nor methane is detected as a product of acetylene reduction; ethylene is not reduced to ethane or methane. The decrease in acetylene during reduction equals the increase in ethylene. The aerobe *Azotobacter* requires aerobic conditions for C_2H_2 reduction, while the anaerobe *Clostridium* reduces acetylene anaerobically. Negligible ethylene is formed in the absence of C_2H_2. Thus, no correction is required for background C_2H_4.

Time Course. Time courses of acetylene reduction by N_2-grown *Azotobacter* and *Clostridium* are shown in figures 9, 10 and 13. The rate of C_2H_2 reduction is constant up to 18 to 20 hours for *Azotobacter* and 6 hours for *Clostridium*. An initial lag is often observed with *Azotobacter*, presumably because of the effect of transfer and dilution. Addition of 40 mM NH_4Cl to N_2-grown cells decreases the rate of C_2H_2 reduction by 95 % after 4 hours (fig 10). This inhibition is in contrast to the effect on N_2ase *in vitro*. The C_2H_2 reduction assay offers a potent method to further define the relationship of N_2 and fixed nitrogen compounds to induction and repression of N_2ase. The results with *Azotobacter* in figure 9 permit calculation of a correlation between C_2H_2 reduction and N_2 fixation. Ethylene formation stops when 0.2 moles of C_2H_4 have been formed for each mole of O_2 initially

Table VII. *Distribution of and Requirements for C_2H_2 Reduction by Cultures of* Azotobacter *and* Clostridium

One ml of culture in early log phase was aseptically added to 4 ml of its nitrogen-free growth media for N_2-grown bacteria and its nitrogen supplemented media for NH_3- or urea-grown bacteria in a sealed incubation vessel of 40 ml total volume containing the indicated gas phase. Incubation time, 16 hr, temperature, 30°. Gas phase assayed chromatographically on ester-amide column.

Organism	Cells ($\times 10^{-6}$)/incubation	Gas phase (atm) A	O_2	C_2H_2	He	μmoles C_2H_4/hr
Azotobacter, N_2-grown	85	0.8	0.2			0.000074
Azotobacter, N_2-grown	85			0.1	0.9	0.030
Azotobacter, N_2-grown	85	0.7	0.2	0.1		1.42
Clostridium, N_2-grown	88				1.0	<0.000001
Clostridium, N_2-grown	88			0.1	0.9	1.05
Azotobacter, N_2-grown	125	0.7	0.2	0.1		1.025
Azotobacter, Urea-grown	240	0.7	0.2	0.1		0.0009
Clostridium, N_2-grown	115			0.1	0.9	2.33
Clostridium, NH_3-grown	210			0.1	0.9	0.12

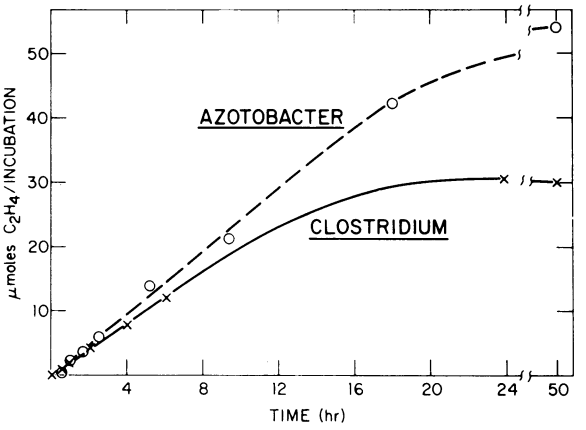

FIG. 9. Time course of $C_2H_2 \rightarrow C_2H_4$ reduction by cultures of N_2-grown *A. vinelandii* and *C. pasteurianum*. Incubation system, table VII; liquid volume, 5 ml; gas volume, 35 ml; temperature, 30°; gas phase, 0.1 atm C_2H_2, 0.2 atm O_2, 0.7 atm A for *Azotobacter* and 0.1 atm C_2H_2, 0.9 atm He for *Clostridium*; *Azotobacter*, 250 × 10^6 cells; *Clostridium*, 110 × 10^6 cells. Points represent averages of 3 samples.

present. Based on aerobic oxidation of glucose, 1.25 moles of ethylene are formed per mole of glucose oxidized. On the basis of one mole of N_2 fixed per 3 to 4 moles of C_2H_2 reduced, 0.3 to 0.4 mole of N_2 would be fixed per mole of glucose oxidized. This calculated ratio of N_2 fixed per glucose oxidized, based on C_2H_2 reduction, is in reasonable agreement with reported experimental values based on direct measurements of N_2 fixation (35).

Cell Number. A linear relationship exists between cell number and acetylene reduced (table VIII). Ethylene formation measured after 16 hours

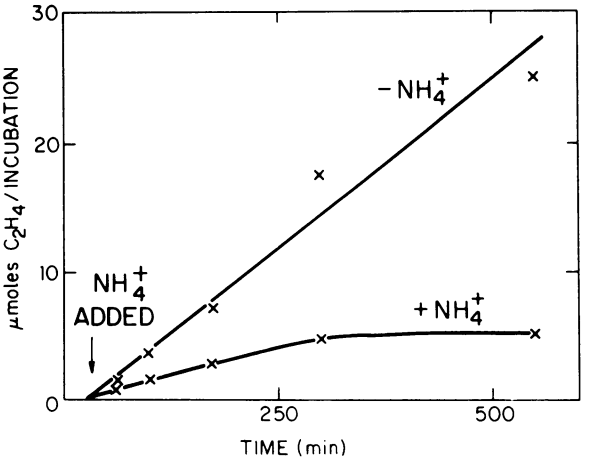

FIG. 10. Effect of NH_4Cl on time course of $C_2H_2 \rightarrow C_2H_4$ reduction by a culture of N_2-grown *A. vinelandii*. Incubation system, figure 9; 300 × 10^6 cells; NH_4Cl added to 3 flasks at 30 min to produce 40 mM NH_4^+ while no addition was made to 3 control flasks.

Table VIII. *Cell Dilution and C_2H_2 Reduction by* Azotobacter *Culture*

Incubation system, table VII, for N_2-grown *Azotobacter*; incubation time, 16 hr; temperature, 30°; gas phase, 0.1 atm C_2H_2, and A:O_2 (0.8:0.2) to 1 atm. Culture serially diluted and cells in original culture counted in a hemocytometer. Ethylene assayed gas chromatographically on ester-amide column.

Cells/incubation	mμmoles C_2H_4/hr incubation	μμmoles C_2H_4/hr·cell
9,250,000	183	0.020
1,550,000	34	0.022
255,000	6.0	0.024
43,000	0.875	0.021
7,000	0.106	0.015

of incubation at 30° is 0.02 μμmole per hour per *Azotobacter* cell over a 1000-fold range of cell concentration. The extreme sensitivity of the C_2H_2-C_2H_4 assay is indicated; theoretically, as few as 2 to 3 cells produce sufficient C_2H_4 for detection by the H_2-flame ionization system.

Km of C_2H_2. Acetylene saturation of N_2-grown clostridial cells occurs between 0.025 and 0.1 atmosphere (fig 11a), and even 0.5 atmosphere is not inhibitory (fig 11b). The saturation concentration is similar for N_2-grown *Azotobacter*, but these cells

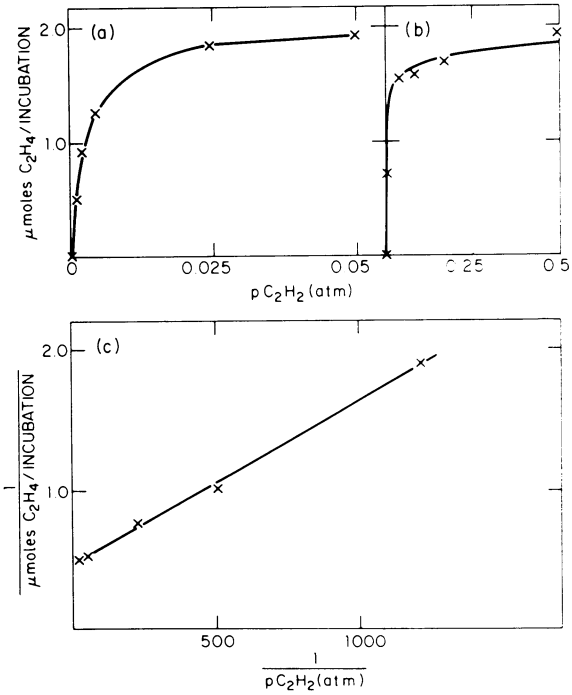

FIG. 11. a) and b). Ethylene formation from C_2H_2 as a function of pC_2H_2 by culture of N_2-grown *C. pasteurianum*, and c) plot of reciprocal velocity *versus* reciprocal pC_2H_2 for determination of *Km* of C_2H_2. Incubation system, table VII; liquid volume, 5 ml; gas phase, indicated pC_2H_2 plus He to 1 atm; points represent averages of 3 incubations.

show an as yet unexplained increase in rate of C_2H_2 reduction at 0.2 and 0.5 atmosphere of C_2H_2. A plot of reciprocal pC_2H_2 *vs.* reciprocal rate of C_2H_2 reduction by clostridial cells is shown in figure 11c. The range of Michaelis constants with *Clostridium* is 0.003 to 0.008 atmosphere C_2H_2 with an average of 0.006, while that for *A. vinelandii* incubated at 0.1 atmosphere or less C_2H_2 is 0.003 to 0.006 atmosphere with an average of 0.005.

Activation Energy of C_2H_2 Reduction. The effect of incubation temperature on C_2H_2 reduction by clostridial cells was determined over the range 10° to 35°. A close analogy with the *in vitro* results on N_2ase is observed which suggests that the limiting factor in growth may be N_2ase activity, and furthermore that this is related specifically to a property of the N_2ase enzyme *per se,* rather than to reactions which furnish energy or reductant to the enzyme. An Arrhenius plot of the cellular activities is shown in figure 12. Results from 20° to 35° form a linear plot with a calculated activation energy of 13 to 15 kcal/mole; results from 10° to 20° are not co-linear with those from 20° to 35°, and a much higher activation energy, approximately 50 kcal/mole, is estimated for the lower temperatures.

C_2H_2 Reduction and N_2 Fixation. Acetylene reduction by N_2-grown *Azotobacter* cultures was compared with N_2 fixation and increase in OD by identical cultures incubated with air under the same conditions. In all cases the 3 parameters measured showed parallel increases with time of incubation. The late-log phase culture (fig 13) did not reduce

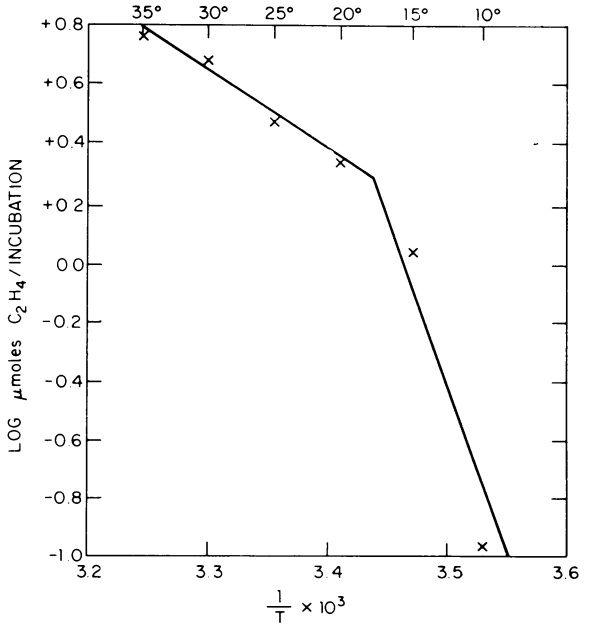

FIG. 12. Arrhenius plot of $C_2H_2 \rightarrow C_2H_4$ by culture of N_2-grown *C. pasteurianum* in the range of 10° to 35°. Incubation system, table VII; liquid volume, 5 ml; gas volume, 35 ml; temperature as indicated; time, 1 hr; gas phase, 0.1 atm C_2H_2, 0.9 atm He.

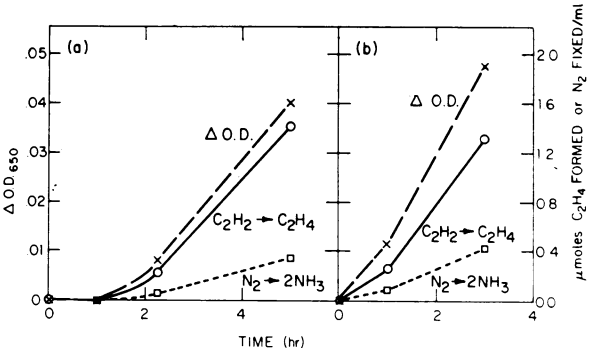

FIG. 13. Acetylene reduction, ΔOD_{650} and N_2 fixation by culture of N_2-grown *A. vinelandii* in a) late log phase, and b) early log phase of growth. Incubation system, table VII; liquid volume, 40 ml; gas volume, 290 ml; temperature, 30°; gas phase, 0.1 atm C_2H_2, 0.2 atm O_2, 0.7 atm A. Initial OD_{650} of late log phase culture after dilution with fresh media was 0.087, while that of early log phase culture was 0.112. Samples of gas phase and culture were analyzed for C_2H_2 and C_2H_4 by gas chromatography and for fixed nitrogen by Kjeldahl analysis at indicated times.

C_2H_2, fix N_2, nor increase in optical density during the initial hour of incubation; in contrast the early log phase culture (fig 13) showed uniformly positive responses during the initial hour. The ratio of moles of N_2 fixed to moles of C_2H_4 formed is 3 to 4.5.

Distribution of C_2H_2-Reducing Activity. The absence of significant C_2H_2-reducing activity in a variety of organisms grown under non-N_2-fixing conditions further establishes the validity of the relationship between C_2H_2-reducing and N_2-fixing ability. Organisms tested included *Clostridium butyricum* under anaerobic conditions on complete

Table IX. *C_2H_2 Reduction by Selected N_2-Fixing and non-N_2-Fixing Bacterial Cultures*

One ml of indicated culture in log phase of growth added to 4 ml of its respective media. Total volume of incubation vessel, 40 ml; gas phase, 0.1 atm C_2H_2, 0.9 atm He for anaerobes, 0.1 atm C_2H_2, 0.2 atm O_2, 0.7 atm A for aerobes; incubation time, 60 min; temperature, 30°. Ethylene assayed gas chromatographically on ester-amide column. Similar results obtained with 0.01 atm C_2H_2.

Organisms	OD_{650}mμ of culture	μmoles C_2H_4/ hr•incubation	N_2-fixing ability
Azotobacter vinelandii	0.30	1.58	+
Clostridium pasteurianum	0.19	1.62	+
Rhizobium japonicum	0.3	0.0002	—
R. melliloti	0.3	<0.00004	—
R. leguminosarum	0.3	<0.00004	—
R. sp. (ATCC 10317)	0.3	<0.0002	—
R. trifolii	0.3	<0.00004	

medium and *Lactobacillus leichmanii, Bacillus subtilis, Bacillus cereus* var. *mycoides, Serratia marcescens, Escherichia coli, Streptococcus lactis, Saccharomyces cerevisiae, Pseudomonas aeruginosa, Pseudomonas fluorescens, Aerobacter aerogenes, Staphylococcus epidermidis, Sarcina lutea, Spirillum itersonii, Proteus vulgaris, Alcaligenes faecalis,* and *Rhodospirillum rubrum* under aerobic conditions on complete medium. All had <0.1 % of the C_2H_2-reducing activity of N_2-grown *A. vinelandii* or *C. pasteurianum.* The absence of C_2H_2-reducing activity (<0.02 % of the *Azotobacter* or clostridial activity) among the various *Rhizobia* grown on the indicated medium (see under Methods) is specifically emphasized, since these data are the most sensitive indication of the inability of cultures of these organisms to fix N_2 (table IX).

C_2H_2 Reduction by Selected Biosphere Samples Assayed in situ. An effective procedure for the *in situ* assay of N_2-fixing activity of the biosphere *via* the C_2H_2–C_2H_4 assay is described in this section. The process is used: 1) to assess requirements, reproducibility, sample variability, and environmental factors that influence C_2H_2-reducing activity of field-grown soybeans, 2) to quantitate C_2H_2-reducing activity of field-grown soybeans through one growing season, and 3) to determine C_2H_2-reducing activities of free-living organisms in the soil and in the hydrosphere. These results represent the first report of the systematic use of C_2H_2 reduction to estimate N_2 fixation during an entire growing season. Since results were obtained during a single season, it is obvious that assays in future seasons will be required to firmly define patterns of N_2 fixation and to establish the effects of variable environmental conditions. Field-grown soybeans were selected for this test because of their agricultural significance. Other biosphere samples were selected to test the universality of the C_2H_2–C_2H_4 assay for determination of N_2 fixation.

Process of in situ *C_2H_2–C_2H_4 Assay.* The 10 steps involved in our *in situ* C_2H_2–C_2H_4 assay process are outlined in figure 14. All the steps are designed to minimize sample alteration and to establish in the assay chamber a micro-ecosystem that is identical in temperature, moisture, porosity, *etc.* to the macro-ecosystem from which the sample is selected. Samples may consist of nodulated plant roots, plant root soil bores, soil bores, or hydrosphere.

Nodulated legumes, after decapitation to eliminate possible background ethylene formation and to decrease the total sample size, were sampled by removal of the complete root system with attached nodules or by a soil bore directly over the tap root. Detached nodules have been used in some preliminary work (23, 33, 34, 36), but they are less active and their collection is time-consuming, particularly in the case of legumes with small nodules, *e.g., Medicago sativa.* We recommend the nodulated root system over the root soil bore system because of: 1) the heterogeneous distribution of nodules per plant and of nodulated plants sown in rows, 2) the injury to nodules by the soil borer, and 3) the lower activity observed with the soil bore technique (fig 18c and 19c). Soil for free-living N_2 fixation is sampled with a soil borer (1-inch × 15-inch graduated in 3-inch steps). Immediately after collection, samples (45 ml) are transferred to the assay chamber, a 50 ml syringe. The diameter of the assay chamber matches the diameter of the soil bore to promote effective gas exchange, addition and removal. Advantages of this assay chamber include: 1) sufficient size to accommodate a representative sample of a nodulated root or 45 ml soil bore plus gas phase, 2) effective and rapid replacement of air with 20 ml additions of desired aerobic [A:O_2(0.8:0.2)], photosynthetic [A:O_2:CO_2(0.8:0.2:0.001)] or anaerobic (A or He) gas phase (step 3), 3) uniform and rapid mixing of 20 ml of C_2H_2 gas mixture (0.2 atm C_2H_2 plus aerobic or anaerobic gas to 1 atm) with sample (steps 5 and 6), and 4) removal and mixing of gas phase at the end of the incubation (step 8), which precludes the requirement for sample inactivation. The determination of C_2H_2 as well as C_2H_4 can provide a useful internal standard for detection and correction of gas leaks, elimination of faulty assays and determination of gas volume of sample in the case of soil bores. Gas volume of soil bores was estimated as 50 % of the volume of the soil bore in the results reported here. It is emphasized that C_2H_2 is a very explosive gas and strict safety measures must be taken to eliminate the possibility of ignition (no smoking) during all steps involving C_2H_2. Observed C_2H_2-reducing activity is converted to a calculated N_2-fixing activity on the basis of the ratio of their electron requirements, *i.e.,* 2 electrons for C_2H_2 reduction and 6 electrons for N_2 reduction. A ratio in the area of 3 to 4.5 is compatible with results of C_2H_2 reduction and N_2 fixation reported in the previous sections. The process described appears to be effective, simple and rapid. More than 450 samples per day have been routinely collected and assayed in the field (steps 1–8) and over 80 samples per hour have been incubated (steps 2–8).

C_2H_2 Reduction by Field-Grown Soybeans—Requirements, Distribution, and Product. Root soil bores or nodulated roots of field-grown soybeans reduced C_2H_2 to C_2H_4 (table X). Neither C_2H_6 nor CH_4 was detected and C_2H_4 was not reduced to C_2H_6 or CH_4. Negligible C_2H_4 (<0.001 % of that with C_2H_2) was formed in the absence of C_2H_2. Root soil bores containing no nodules possessed only a very low level of C_2H_2-reducing activity (0.01–0.02 % of bores containing nodules), equivalent to that of soil bores made 19 inches from the soybean rows or of soil collected around the nodulated roots. Samples containing nodules but not possessing C_2H_2-reducing activity were always found to contain only white nodules. Aerobic conditions are required

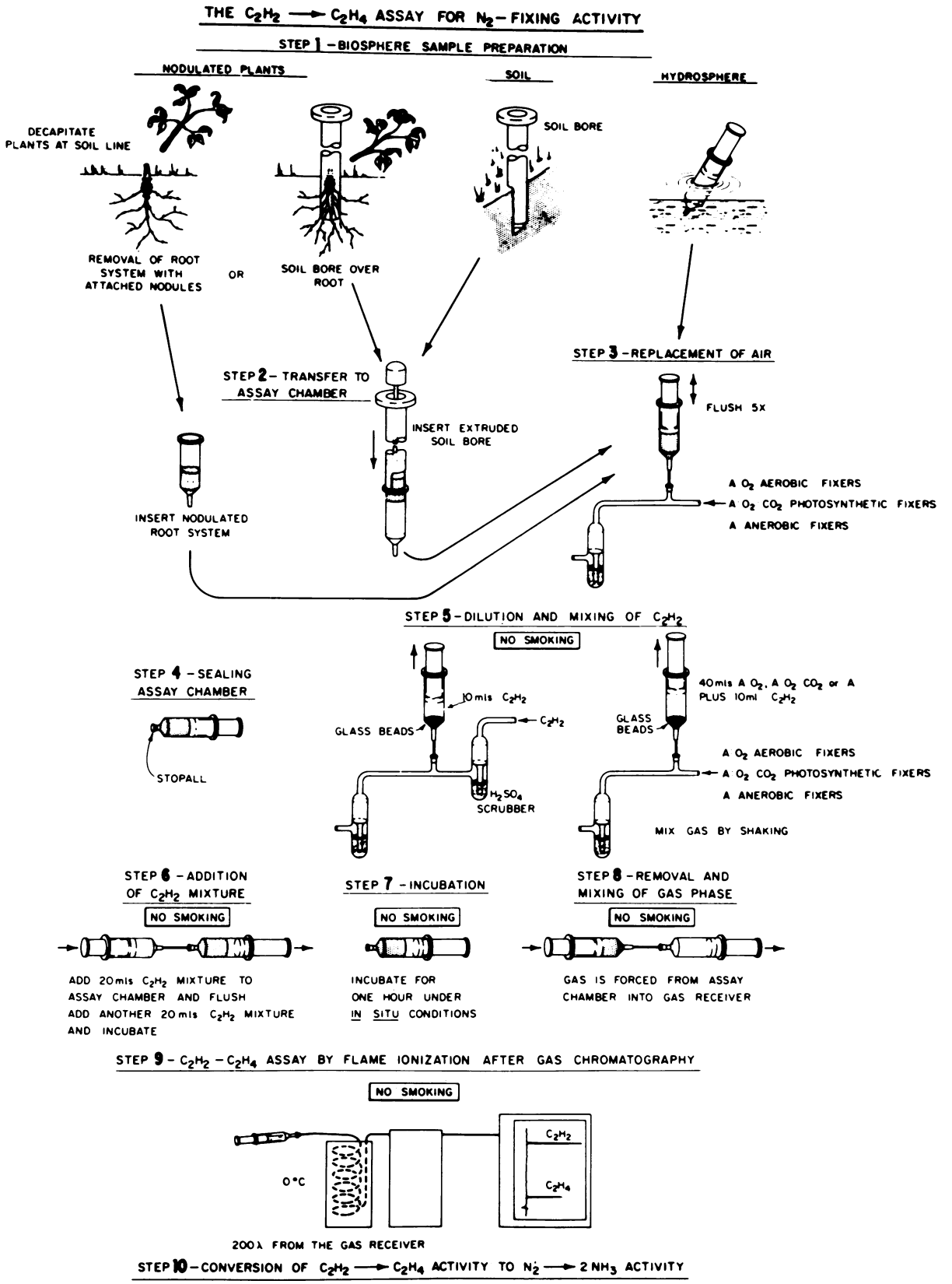

FIG. 14. Steps in $C_2H_2 \rightarrow C_2H_4$ assay for N_2-fixing activity of the biosphere, including samples of nodulated plants, soil, or hydrosphere.

Table X. *Distribution of C_2H_2-Reducing Activity in Soybean Field*

Samples collected between 8 to 9 AM and immediately assayed as outlined in figure 14. Gas phase, 0.1 to 0.2 atm C_2H_2 plus indicated gases A:O_2 (0.8:O_2), or He to 1 atm; root soil bore volume, 45 ml; total gas phase volume, 40 ml; incubation time, 1 hr.

Sample	Gas phase	μmoles C_2H_4/day•sample	mμmoles C_2H_4/day •mg fr wt nodule	mg fr wt nodule
Root soil bore	A:O_2	0.001	0.001	1030
Root soil bore	He:C_2H_2	1.87	3.74	503
Root soil bore	A:O_2:C_2H_2	0.026	...	1030
Root soil bore	A:O_2:C_2H_2	171	168	0
Nodulated root	A:O_2:C_2H_2	195	172	1114
Soil around nodulated root	A:O_2:C_2H_2	0.035	...	0
Soil bore between rows 0–4″ deep	A:O_2:C_2H_2	0.033	...	0
4–8″ deep	A:O_2:C_2H_2	0.030	...	0
8–12″ deep	A:O_2:C_2H_2	0.028	...	0

for C_2H_2 reduction (table X, ref. 23). Failure to replace air (N_2) with A:O_2 results in a 10 to 20% decrease in C_2H_2 reduction (table XI).

Time Course. The rate of C_2H_2 reduction by nodulated roots or root soil bores of soybeans is constant up to 60 minutes (fig 15) with the standard system described in figure 14. However, it is recommended that heavily nodulated roots be assayed for a shorter time (30 min) since the rate for such samples decreases shortly after 60 minutes. This decrease is presumably due to O_2 depletion, since samples which were reflushed with A:O_2 and regassed with the C_2H_2 mixture showed activities during a 1 to 2 hour incubation that were comparable to those observed during a 0 to 1 hour incubation (tables XI, XIII).

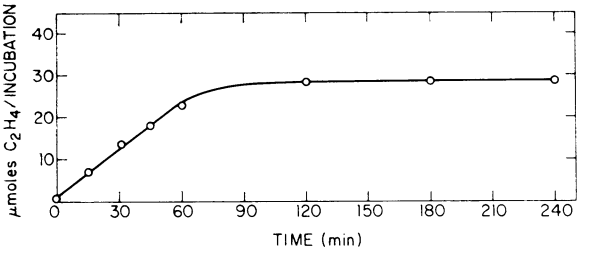

FIG. 15. Time course of $C_2H_2 \rightarrow C_2H_4$ reduction by nodulated soybean roots. Incubation system, figure 14, except that analyses were made from 0 to 240 min. Glass beads were added to assay chambers to facilitate mixing of gas phase.

Interval Between Sampling and Assay. Nodulated roots and root soil bores of soybeans were assayed at 0, 2, 6.5, and 13 hours after sampling (fig 16). Values are expressed on the basis of nodular efficiency, mμmoles C_2H_4 per mg fr wt nodule per day, in order to compensate for the variable nodulation of samples. The results indicate the importance of a minimum interval (0–2 hr) between sampling and assay in order to obtain values which are representative of the *in situ* activity. Consequently, all the results reported in this paper unless otherwise indicated were obtained from assays initiated within 30 minutes after collection.

Sample Variability and Reproducibility. The sample variability with respect to C_2H_2-reducing activity of field-grown soybeans is shown in table

Table XI. *Effect of Air on C_2H_2-Reducing Activity of Soybeans*

Samples were collected between 8 to 9 AM and assayed from 0 to 1 and again from 1 to 2 hr as outlined in figure 14. Air (N_2) replaced A:O_2 where indicated.

Sample	Gas phase	μmoles C_2H_4/day•sample	mμmoles C_2H_2/day •mg fr wt nodule	mg fr wt nodule
Nodulated root				
0–1 hr	A:O_2:C_2H_2	659	197	3333
1–2 hr	A:O_2:C_2H_2	750	225	3333
0–1 hr	A:O_2:C_2H_2	410	91	4511
1–2 hr	Air:C_2H_2	328	73	4511
Root soil bore				
0–1 hr	A:O_2:C_2H_2	185	79	2340
1–2 hr	A:O_2:C_2H_2	190	81	2340
0–1 hr	A:O_2:C_2H_2	289	142	2047
1–2 hr	Air:C_2H_2	266	130	2047

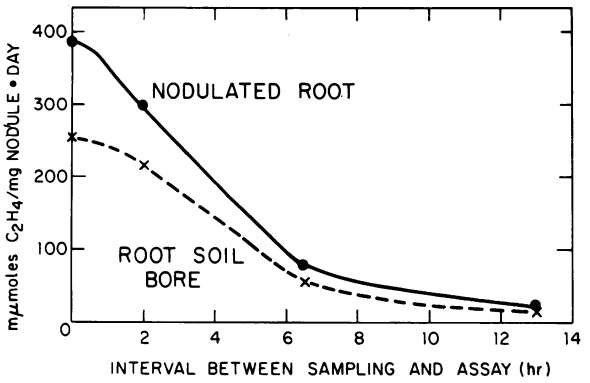

FIG. 16. Reduction of $C_2H_2 \rightarrow C_2H_4$ by nodulated soybean root or soybean root soil bore assayed at various times after collection between 8 to 9 AM. Incubation system, figure 14. Each point represents the average of 5 individual samples.

XII. The standard deviation of C_2H_2-reducing activity of root soil bores or nodulated roots collected at 15, 25, 29, and 44 days post-flowering is 25 to 35 % while that of nodular efficiency is 17 to 22 % for root soil bores and 5 to 10 % for nodulated roots. Thus, expression of activity on the basis of nodular efficiency substantially decreases the variability due to differences in nodule weights.

The excellent reproducibility of C_2H_2-reducing activity of field-grown soybeans is shown in table XIII. Samples collected at various times after flowering show C_2H_2-reducing activities during a 1 to 2 hour assay that are 89 to 107 % of the respective activities determined during a 0 to 1 hour assay.

Km of C_2H_2. Nodulated roots of soybeans are saturated by 0.025 to 0.2 atmosphere C_2H_2 (fig

Table XII. *Sample Variability of Field-Grown Soybeans for C_2H_2 Reduction*

All samples collected from different areas of the same field between 8 to 9 AM on indicated day and assayed immediately as described in figure 14. Each value represents average of 5 samples.

Sampling time (days post-flowering)	μmoles C_2H_4/ day•sample	mμmoles C_2H_4/ mg fr wt nodule•day	mg fr wt nodule/ sample
Root soil bores			
15	33	94	355
	47	72	659
	68	84	812
	43	62	701
Avg	48	79	632
25	105	101	1035
	190	153	1238
	90	180	501
	116	149	776
Avg	125	146	888
Nodulated root			
29	513	176	2908
	514	144	3577
	785	166	4731
Avg	604	162	3739
44	751	226	3319
	412	242	1697
Avg	582	234	2508

Table XIII. *Reproducibility of C_2H_2-Reducing Activity of Field-Grown Soybeans*

Soybean root soil bores or soybean nodulated roots collected between 8 to 9 AM and assayed as in figure 14, from 0 to 1 hr and again from 1 to 2 hr after collection. Each value is average of 5 individual samples.

Sampling time (days post-flowering)	μmoles C_2H_4/sample•day		mμmoles C_2H_4/mg fr wt nodule•day		mg fr wt nodule/ sample
	0–1 hr	1–2 hr	0–1 hr	1–2 hr	
Root soil bore					
0	12	12	74	79	158
0	8	9	71	83	113
2	12	11	65	58	191
44	177	166	56	53	3140
Nodulated root					
44	752	742	226	223	3319

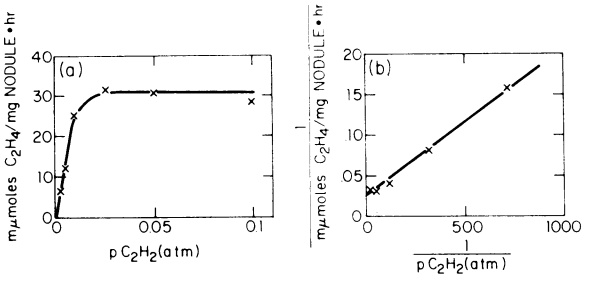

FIG. 17. a) Ethylene formation from C_2H_2 as a function of pC_2H_2 by nodulated roots of soybeans, and b) plot of reciprocal velocity *versus* reciprocal pC_2H_2 for determination of K*m* of C_2H_2. Incubation system, figure 14.

17a); soybean root soil bores showed identical activities when incubated with a C_2H_2 gas mixture of 0.1 to 0.4 atmosphere C_2H_2. Activity was decreased by 0.5 atmosphere C_2H_2. This decrease might be due to direct inhibition by C_2H_2 or to indirect inhibition by the concomitant depletion of O_2. An average K*m* of 0.007 atmosphere C_2H_2 is obtained from a plot of reciprocal pC_2H_2 *vs.* reciprocal rate of C_2H_4 formation. This value is somewhat lower than that reported for excised soybean nodules (23).

Temperature. The effect of temperature of incubation on C_2H_2 reduction by nodulated roots or root soil bores of soybeans was less pronounced than observed with either N_2ase preparations or bacterial cultures. Activity was lower at 10° to 15° and possibly at 35° than at 20° to 30° (table XIV). In contrast, preliminary results suggest that the temperature of growth has a more marked effect on C_2H_2-reducing activity. Nodulated roots from plants maintained in growth cabinets for periods of 1 to 14 days at 30° had only 10 to 20% of the C_2H_2-reducing activity of those at 20°.

C_2H_2-Reducing Activity and Calculated N_2 Fixation During One Growing Season. Acetylene-reducing activity of field-grown soybeans was determined during a complete growth and maturation cycle. Both the root soil bore (fig 18) and nodulated root (fig 19) techniques were used. Samples were collected on 41 different days for the root soil bore assays and on 27 different days for the nodulated root analyses. The C_2H_2-reducing activity was determined as described in figure 14 utilizing 1 hour incubations. Results are expressed on a 24 hour basis (fig 18a and 19a). The fresh weight of nodules per sample was tabulated (fig 18b and 19b), and the nodular efficiency of C_2H_2 reduction, expressed as mμmoles C_2H_4 formed per mg fresh weight nodule per day, was calculated (fig 18c and 19c). The averages of all analyses on an individual day and during each week are shown.

Nitrogen fixation, as measured by the C_2H_2–C_2H_4 assay, was found to parallel the nitrogen demand of

Table XIV. *Effect of Temperature on C_2H_2 Reduction by Soybeans*

Soybean root soil bores or nodulated roots collected between 8 to 9 AM, flushed with A:O_2 as described in figure 14, equilibrated for 10 min in a water bath at indicated temperature, then incubated for 1 hr after addition of C_2H_2 mixture. Values are the averages of 3 nodulated roots or 5 root soil bores.

Incubation temp	Nodulate root	Root soil bore
deg	*mμmoles C_2H_4/mg fr wt nodule•day*	
10	29	...
15	86	...
20	214	156
25	176	206
30	250	188
35	151	151

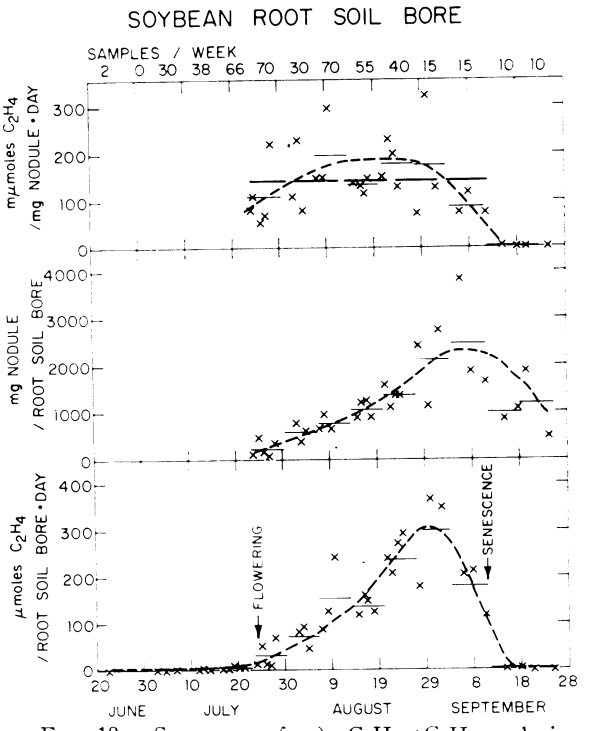

FIG. 18. Summary of a) $C_2H_2 \rightarrow C_2H_4$ reducing activity, b) mg fr wt nodule, and c) mμmoles $C_2H_2 \rightarrow C_2H_4$ per mg fr wt nodule per day by soybean root soil bores at various stages from initiation of activity through flowering and maturation to loss of activity. Incubation system, figure 14; root soil bore volume, 45 ml; gas phase volume, 40 ml. Flowering indicated by initial bud opening and senescence indicated by initial yellowing of leaves. The average of all samples assayed each day (x), during each week — and the average of mμmoles $C_2H_2 \rightarrow C_2H_4$ per mg fr wt nodule per day for the period from flowering to senescence (— — —) are recorded. The number of soil bores of soybean roots assayed each week is recorded. Samples were collected between 8 to 9 AM except for July 27 to 28 and August 9 to 10 when samples were collected at various times as specified in figure 20. Samples were assayed immediately under *in situ* conditions of temperature and moisture. Assay time, 1 hr; results expressed on 24 hr basis.

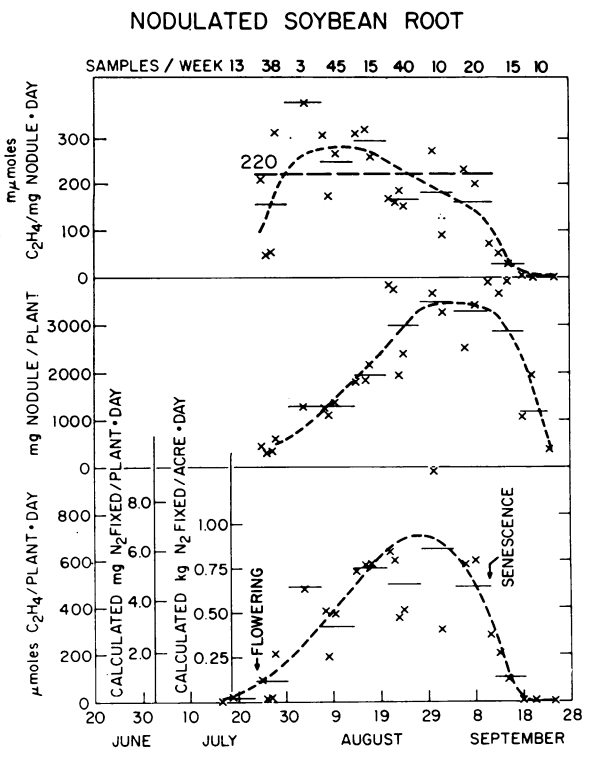

FIG. 19. Summary of a) $C_2H_2 \rightarrow C_2H_4$ reducing activity, b) mg fr wt nodule and c) mμmoles $C_2H_2 \rightarrow C_2H_4$ per mg fr wt nodule per day by nodulated roots of field-grown soybean plants at stages of development similar to those in figure 18. Incubation system, figure 14; gas phase volume, 40 ml. The average of all samples assayed each day (x), each week (——), and the average mμmoles $C_2H_2 \rightarrow C_2H_4$ per mg fr wt nodule per day for the period from flowering to senescence (— — —) are shown. The number of nodulated soybean roots assayed each week is recorded. Sample collection and assay conditions as described in figure 14. Mg N_2 fixed per plant per day and kg N_2 fixed per acre per day are calculated on the theoretical basis of one-third N_2 reduced per C_2H_2 reduced.

the plant. Low C_2H_2-reducing activity occurred until macroscopic flowering was observed, although activity could be detected as early as 32 days before this time; the utilization of residual nitrogen fertilizer during this period may have suppressed N_2-fixing activity to some extent. Following flowering the C_2H_2-reducing activity increased continuously, reflecting the increasing nitrogen requirement for pod formation and filling. Average weekly activity increased from 30 to 299 μmoles C_2H_4 formed per root soil bore per day or 84 to 650 μmoles C_2H_4 formed per plant root per day. The average weekly nodule weight increased from 244 to 2453 mg per root soil bore or 457 to 3478 mg per nodulated plant root. After pod filling was complete the C_2H_2-reducing activity rapidly declined. The decline in nodule weight following senescence lagged behind that of C_2H_2-reducing activity.

The nodule efficiency was relatively constant during the period from flowering to senescence (fig 18c and 19c). The average weekly efficiencies varied from 88 to 196 mμmoles C_2H_4 formed per mg fresh weight nodule per day for root soil bores and from 156 to 378 for nodulated roots. The average nodule efficiency from flowering to senescence was 142 mμmoles C_2H_4 formed per mg fresh weight nodule per day for root soil bores and 220 for nodulated roots.

Summation of the weekly averages of C_2H_2-reducing activity indicates that 22.5 mmoles of C_2H_4 could be formed per plant per season (fig 19a). The calculated N_2 fixation per acre per season is 30 to 33 kg of nitrogen based on: 1) the C_2H_2–C_2H_4 assay, 2) 142,000 plants per acre, and 3) a theoretical conversion factor of one-third N_2 fixed for each C_2H_4 formed.

$$\frac{22.5 \text{ to } 25}{3} \times \frac{28}{10^6} \times 142{,}000 = 30 \text{ to } 33 \text{ kg } N_2 \text{ fixed per acre per season}$$

This calculated value was determined during development of the C_2H_2–C_2H_4 assay and with samples (except for 2 occasions) collected between 8 to 9 AM, a period which may represent less than maximal activity (fig 20). However, this value is in excellent agreement with the average value of 38 kg

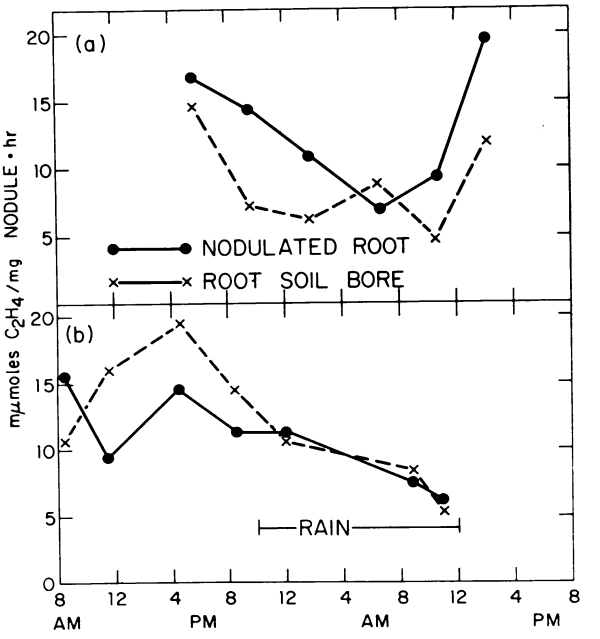

FIG. 20. Diurnal variation of $C_2H_2 \rightarrow C_2H_4$ reduction by nodulated soybean roots and soybean root soil bores collected in the field at indicated times and assayed immediately. a) Samples 3 to 4 days post-flowering, and b) 16 to 17 days post-flowering. Incubation system, figure 14. Each point represents the average of 5 samples. Heavy rainfall occurred during the night and second day of experiment in b).

of nitrogen fixed per acre reported for Kjeldahl and ^{15}N analyses of N_2 fixation by soybeans (35). This correlation provides support for the quantitative reliability of the C_2H_2–C_2H_4 assay performed as outlined in figure 14.

Diurnal Variation. The diurnal variation of C_2H_2-reducing activity of field-grown soybeans was determined at 3 to 4 (fig 20a) and 16 to 17 (fig 20b) days post-flowering. Activities expressed as mμmoles C_2H_4 formed per mg fr wt nodule per hour appear to be maximal for samples collected from noon to 8 PM and minimal for those collected from midnight to 8 AM. Thus, a close relationship between light and N_2-fixing activity is suggested. The effect of light on C_2H_2-reducing activity was further demonstrated with nodulated roots of soybean plants in growth cabinets (fig 21). Control plants maintained on a 16 hour light and 8 hour dark cycle did not show a marked diurnal variation, while experimental plants showed a rapid decline to 30 % of control activity after 17 hours of total darkness, but still had 15 % of control activity after 64 hours of darkness. The initial decline may reflect the depletion of photosynthate, while the residual activity may represent utilization of storage products.

Heavy rainfall eliminated the normal diurnal variation (fig 20b). This effect might be due to increased soil moisture and/or decreased light intensity. Saturation of soybean root soil bores with water decreased their C_2H_2-reducing activities from 170 to 50 mμmoles C_2H_4 per mg fresh weight nodule per day.

Leaf or Pod Removal. Removal of leaves decreased C_2H_2-reducing activity to 12 % of control after 1 day, and this activity was still only 14 % of control at 10 days after leaf removal.

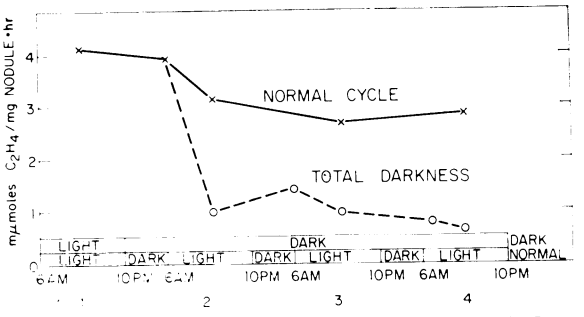

FIG. 21. Effect of light and darkness on $C_2H_2 \rightarrow C_2H_4$ reduction by nodulated soybean roots from plants maintained in plant growth chamber. Incubation system, figure 14. Each point represents the average of 5 individual samples. Control cycle, 6 AM to 10 PM, 24°, with maximum light (see under Methods), and 10 PM to 6 AM, 18°, total darkness. Experimental sample was placed in total darkness with normal temperature cycle at 10 PM of first day of experiment.

Removal of pods at 19 days post-flowering did not alter C_2H_2-reducing activity per plant (fig 22a) or mg fresh weight nodule per plant (fig 22b) during the following 10 days. However, during the same period control plants increased both C_2H_2-reducing activity per plant and mg fresh weight nodules per plant. Thus, the magnitude of N_2 fixation reflects the demands of the plant, specifically the pod in this case, for nitrogen.

Varieties. Acetylene reduction provides a technique for the determination of differences in the N_2-fixing activities of different varieties of legumes. A single exploratory experiment was conducted with

Table XV. *C_2H_2 Reduction by Different Varieties of Field-Grown Soybeans*

Nodulated roots of soybean varieties collected between 2 to 3 PM on the same day (114 days after planting) were assayed immediately for C_2H_2 reduction as indicated in figure 14. Soil temperature, 19 to 20°. C1311-Wabash X C1069-Clark X C1069; and UD 61-1806-EC33243 X D49-249L. Each value represents 5 individual roots.

Variety	Stage of development	μmoles C_2H_4/plant•day	mμmoles C_2H_4 /mg fr wt• nodule day
Early-maturing:			
Verde	Yellow, loss of leaves	1.5	1.1
Adelphia	"	1.1	1.5
Clark	"	1.0	1.8
Intermediate-maturing:			
C1311	" + nodule decay	0.8	4.8
C1278	Initial yellowing	7.0	7.9
Kent	"	22.7	17
Delmar	"	30.2	39
Late-maturing:			
Dare	Green, pods still filling	35.5	57
York	"	59.2	98
UD61-1806	"	136	104
Hill	"	162	119

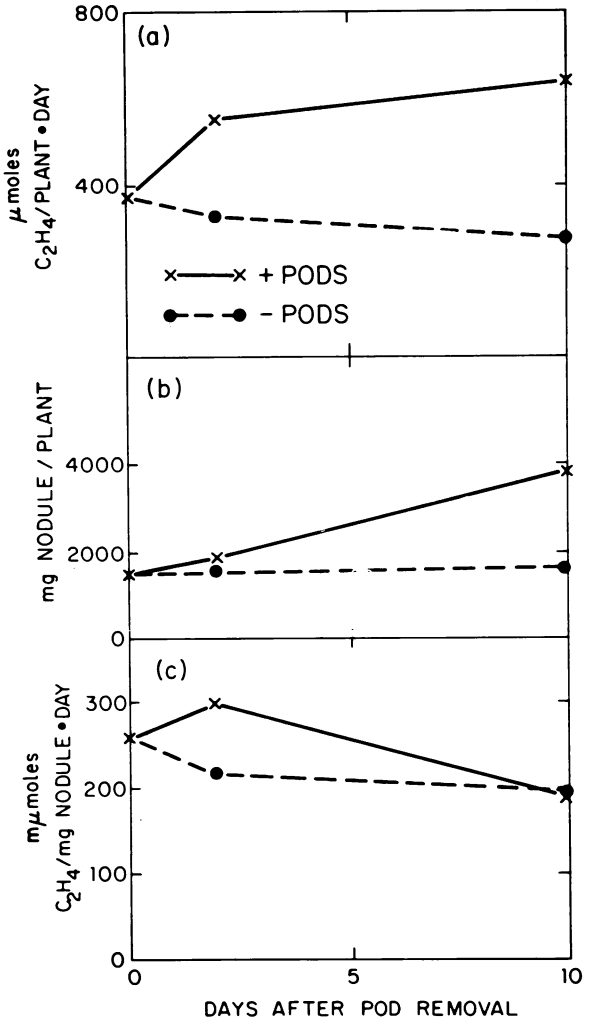

FIG. 22. a to c). Effect of pod removal on $C_2H_2 \rightarrow C_2H_4$ reduction by nodulated soybean root or soybean soil bore. Incubation system, figure 14. Pods were removed 19 days post-flowering.

varieties of soybeans representing different maturation dates. Marked differences found in C_2H_2-reducing activity (table XV) correlated with differences in the stage of maturity at analysis. Varietal differences will be further investigated.

C_2H_2 Reduction by Selected Legumes. Nodulated roots of *Phaseolus vulgaris, Medicago sativa, Arachis hypogea,* and *Pisum sativum* as well as *Glycine max* reduce C_2H_2 to C_2H_4 (table XVI). The nodular efficiencies, mμmoles C_2H_4 formed per mg fresh weight nodule per day, of these legumes were in the range of 106 to 402, similar to those reported by Koch and Evans (23) for excised soybean nodules, but substantially higher than those reported by Sloger and Silver (34) and subsequently by Stewart, Fitzgerald, and Burris (36) for a variety of excised legume and non-legume nodules.

Table XVI. *C_2H_2 Reduction by Selected Legumes*

Nodulated roots of the indicated legumes collected between 8 to 10 AM and immediately assayed as described in figure 14.

Legume	mμmoles C_2H_4/mg fr wt nodule•day	Calculated kg N_2 fixed/acre•day
Glycine max[1]	220	0.60
Phaseolus vulgaris[2]	106	...
Medicago sativa[2]	161	0.47
Arachis hypogea[2]	402	...
Pisum sativum[2]	304	...

[1] Average value from figure 19 for flowering to senescence period.
[2] Samples assayed at flowering.

C_2H_2 Reduction by Free-Living Bacteria in Soil. Nitrogen-fixing activities calculated from the C_2H_2-reducing activities of selected soil samples are tabulated in table XVII. The activities varied over 500-fold between locations and suggest that N_2-fixation by free-living soil bacteria varies from negligible to highly significant. Activities under both aerobic and anaerobic conditions were found; the anaerobic activity was higher in the lower soil levels.

Variations in C_2H_2-reducing activity of the Jordan Fertility Plot samples correlated with nutrient applications. The 4 plots receiving recommended additions of N, P, and K showed similar activities, and the average calculated value of N_2 fixation is 0.51 kg of N_2 fixed per acre per day. Plots receiving less or no nitrogen showed, in general, greater activities. The calculated N_2 fixation of the plot receiving only K is 1.52 kg N_2 fixed per acre per day. This is the highest calculated value of N_2 fixation that we have obtained for soil samples analyzed with the C_2H_2–C_2H_4 assay. Samples from all the limed plots had double the activity of those from all the unlimed plots. Initial experiments with soil to which bacterial cultures were added (*Azotobacter* and *Clostridium*) showed negligible increases in C_2H_2-reducing activity.

Miscellaneous Examples of C_2H_2 Reduction. Nitrogen-fixing activity of a variety of miscellaneous samples of the biosphere, including pond water and rumen contents were assayed by C_2H_2 reduction. Samples of pond water reduced C_2H_2 to C_2H_4 when they contained blue-green algae. Negligible C_2H_4 was formed in the absence of C_2H_2. More extensive studies of C_2H_2 reduction by blue-green algae have been communicated recently (36).

Rumen contents from a fistulated steer reduced C_2H_2 to C_2H_4. Ethylene formation was 10-fold greater under anaerobic than aerobic conditions. Methane formation was markedly decreased in the presence of acetylene. The N_2-fixing activity of a rumen calculated on the basis of the anaerobic results was 10 mg N_2 fixed per rumen per day.

Table XVII. *C_2H_2-Reducing Activity and Calculated N_2 Fixation by Soil Samples*

Soil bores were collected from 12 to 4 PM and immediately assayed as indicated in figure 14. Each value represents the average of 6 samples. Soil bores, 2 × 45 ml for 0 to 6" and 1 × 45 ml for 0 to 3" and 6 to 9".

Sample level	Special treatment	mμmoles C_2H_4/sample•day		Calculated kg N_2 fixed /3" or 6" acre•day	
		Aerobic	Anaerobic	Aerobic	Anaerobic
Chester Co., Pa.					
0-3"	...	31	67	0.0020	0.0043
3-6"	...	27	205	0.0018	0.0130
6-9"	...	27	75	0 0018	0.0048
Georgetown, Del.					
0-3"	. .	310	155	0.020	0.010
6-9"	...	235	940	0.015	0.106
Jordan fertility plots					
0-6"	+K[1]	11,900	...	1.52	...
0-6"	+N[1]	11,500	...	1.47	...
0-6"	+P[1]	8,700	...	1.12	...
0-6"	No fertilizer	8 600	...	1.10	...
0-6"	+P,K	8,740	...	1.02	...
0-6"	+N,P	7,400	...	0.95	...
0-6"	+N,K	5,850	...	0.75	...
0-6"	+N,P,K,	5,700	...	0.73	...
0-6"	Recommended treatment[2]	4,600	...	0.59	...
0-6"	"	4,200	...	0.54	...
0-6"	"	4,100	...	0.53	...
0-6"	"	2,900	...	0.37	...
0-6"	+3(N), as $NaNO_3$ P,K	2,800	. .	0.36	...
0-6"	Excess burned lime	2,260	...	0.29	...
0-6"	+N,P,K	1,400	...	0.27	...
0-6"	Ground bone	1,250	...	0.18	...
0-6"	+3(N) as $(NH_4)_2SO_4$, P,K	1,100	...	0.16	...
0-6"	+Lime	5,850	..	0.75	...
0-6"	—Lime	2,650	...	0.34	...

[1] +K = 100 lb K_2O per acre; +N = 24 lb N per acre; +P = 48 lb P_2O_5 per acre.
[2] Recommended treatment = 115 lb N, 130 lb P_2O_5,130 lb K_2O per acre.

Discussion

The results reported here support the validity of the C_2H_2–C_2H_4 assay as a sensitive and universal analysis for N_2-fixing activity. The advantages of this assay indicate significant broad applications for measurement of N_2 fixation in both laboratory and field investigations. The essential relationship between C_2H_2-reducing activity and N_2-fixing activity is supported and extended to a most convincing degree by studies within and among cell-free extracts, bacterial cultures, and symbionts.

This relationship is firmly established by detailed experiments with *Azotobacter* preparations which lead to the conclusion that C_2H_2 and N_2 evoke identical responses from N_2ase. Convincingly parallel responses are observed with respect to: requirement for ATP and reductant, linear time course, optimum pH, sigmoidal relationship between rate and enzyme concentration, inhibition of H_2 evolution in an amount equivalent in electrons to those used for C_2H_2 or N_2 reduction, competitive inhibition by CO, relative insensitivity to NH_4^+, activation energy of 13 to 15 kcal/mole above 20° and 35 to 50 kcal/mole below 20°, activity in extracts from N_2- but not urea-grown cells, distribution of activity during fractionation, requirement for both the Mo-Fe and Fe protein fractions of N_2ase, and relative inactivity of the clostridial-*Azotobacter* interspecies recombination of the Mo-Fe and Fe protein fractions. These results provide the most complete correlation between N_2 fixation and C_2H_2 reduction to date. Some of these similarities have been reported for cell-free extracts of bacteroids (24, 25) and *C. pasteurianum* (13, 32). The inhibition of ATP-dependent H_2 evolution by *Azotobacter* N_2ase in an amount equivalent to the formation of ethylene establishes the electron-activating reaction of N_2ase as the source of electrons for C_2H_2 reduction, just as for N_2, N_2O and N_3^- reductions (15, 20). The similar competitive inhibitions of N_2 fixation and C_2H_2 reduction by CO provide strong indirect support for the role of the substrate-complexing site of *Azotobacter* N_2ase for both C_2H_2 and N_2 reduction.

Whole cell experiments are completely consistent with the results obtained *in vitro* and demonstrate the parallel C_2H_2–N_2 relationship in *in vivo* analyses. Thus, cultures of *Azotobacter* or *Clostridium* reduce C_2H_2 to C_2H_4 with characteristics similar to fixation of N_2, which include: anaerobic requirement

for *Clostridium* and aerobic requirement for *Azotobacter*, activity in N_2- but not NH_3- or urea-grown cells, time course, and ratio of C_2H_2 reduced to N_2 fixed of 3 to 4.5. In addition, bacterial species without N_2-fixing activity do not possess significant C_2H_2-reducing activity.

Experiments with symbionts establish that the C_2H_2-N_2 correlation is consistently applicable to even these most complex natural N_2-fixing systems. Thus, various legumes also reduce C_2H_2 to C_2H_4 with characteristics similar to fixation of N_2. These include: aerobic requirement, activity only in nodules possessing leghemoglobin, absence of activity in either the root or infecting bacteria, and similar rate of C_2H_4 formation or N_2 fixation per season based on electron requirement.

The characteristics of N_2ase activity, as exemplified by C_2H_2 reduction, are consistent through the entire range of organization studied; results obtained with the most defined *in vitro* system apply with equal validity to even the most complex symbiotic systems. Thus, all systems reduce C_2H_2 to a single significant product, C_2H_4; none reduce substrate C_2H_4; all are saturated by 0.02 to 0.2 atmosphere of C_2H_2; the *Km* values are 0.002 to 0.009 atmosphere, and the calculated activation energies are similar for both N_2ase preparations and bacterial cells with a break in the Arrhenius plots near 20°. A common enzyme, N_2ase, appears to be responsible for C_2H_2 reduction by these diverse systems.

The advantages and disadvantages of the C_2H_2–C_2H_4 assay for N_2 fixation are summarized in table XVIII. The advantages emphasize the superior attributes of this method relative to other assays of N_2 fixation.

Sensitivity of C_2H_4 detection by flame ionization as initially indicated by Hardy and Knight (19) is the critical advantage of the C_2H_2–C_2H_4 assay. The sensitivity of this method is related to other methods of N_2 fixation by the following comparison: the C_2H_2–C_2H_4 method is to the ^{15}N method as the ^{15}N method was to the Kjeldahl method. This sensitivity of the C_2H_2–C_2H_4 assay makes it possible to detect low levels of N_2-fixing activity in biosphere samples, bacterial cultures, or N_2ase preparations, and to investigate variations in N_2-fixing activity with short-term incubations. It is anticipated that the list of N_2-fixing organisms will undergo additions and possibly some deletions because of the C_2H_2–C_2H_4 assay. The only other method with equivalent or greater sensitivity is ^{13}N-incorporation; however, the complex production facilities and short half-life restrict the application of ^{13}N to limited laboratory investigations.

Disadvantages of the C_2H_2–C_2H_4 assay include the indirect nature of the reaction. Although no defined sample has been found with significant C_2H_2-reducing activity and without N_2-fixing activity, the possibility exists for a non-N_2ase-dependent catalysis by biosphere samples of the reduction of C_2H_2 to C_2H_4. The explosive nature of C_2H_2 is emphasized. Laboratories accustomed to utilizing the relatively inert N_2 for ^{15}N or Kjeldahl analysis are cautioned to employ safe practices with C_2H_2.

As recently as 3 years ago Allison (2) found it necessary to state "During the past 50 years much effort has been devoted to the economic evaluation

Table XVIII. *The C_2H_2–C_2H_4 Assay for N_2 Fixation*

Advantages

A) Analytical

Sensitivity. <1 $\mu\mu$mole C_2H_4 per sample is detectable; 10^3 times as sensitive as ^{15}N and 10^6 times as sensitive as Kjeldahl analyses.

Facility. No chemical treatment or manual manipulation of product required, *c.f.* ^{15}N and Kjeldahl analyses which require several time-consuming chemical conversions. Both product and unused reactant are simultaneously recovered in a single, easily secured sample of the gas phase which is directly analyzed.

Specificity. C_2H_4 is separated from CH_4, C_2H_2, C_2H_6.

Internal Standard. C_2H_2 is a natural internal standard and its measurement requires no additional steps.

Storage Stability of Product. C_2H_4 stable indefinitely in simple containers at ambient temperature.

Simplicity. *In situ* assay process and gas chromatography are simple techniques and can be performed by technicians *c.f* ^{15}N mass spectrometric analysis. Few and simple calculations convert raw data to activity values.

Economy. Substrate, assay hardware, and gas chromatographic equipment are inexpensive *c.f.* mass spectrometric equipment and $^{15}N_2$ cost.

Rapidity. Ten complete assays (fig 1) per man hour in the field. Twenty complete gas chromatographic analyses per man hour in the laboratory.

Mobility. All required equipment sufficiently rugged and portable to permit use of a mobile analytical unit.

Sample Conservation. The test material is not sacrificed by atmospheric sampling and sequential samples can be obtained from a single incubation.

B) Biological

Characteristics of $C_2H_2 \rightarrow C_2H_4$ parallel those of $N_2 \rightarrow 2NH_3$.

Universality. N_2ase preparations, and N_2-fixing bacteria, blue-green algae and symbionts reduce $C_2H_2 \rightarrow C_2H_4$, while non-$N_2$-fixing preparations or organisms do not.

Quantitative Relationship of C_2H_2 reduced to N_2 fixed of 3 to 4.

Saturation by a low $pC_2H_2 - Km$ (C_2H_2) of 0.003-0.008 atm *c.f.* $Km(N_2)$ of 0.02 to 0.16 atm.

Specificity of Reaction Product. No significant product other than C_2H_4.

Low Background. Negligible C_2H_4 formed in the absence of C_2H_2 *c.f.* the natural background of ^{15}N.

Metabolic Stability of Product. C_2H_4 is not metabolized *c.f.* the metabolic conversions of inorganic and organic forms of nitrogen.

Disadvantages

Indirect Nature of Reaction. The possibility exists for non-N_2ase catalysis of this reduction by samples of the biosphere.

Explosive Nature of C_2H_2. C_2H_2 is a highly explosive gas *c.f.* N_2 which is relatively inert.

of free-living nitrogen-fixing micro-organisms in soils, but we are nearly as much in the dark now----. There is no sound base upon which to make direct estimate----", while only a year ago the Subcommittee of Production Processes of the United States National Committee for the International Biological Program (37) suggested the following immediate action with respect to nitrogen fixation: "Providing means for the use of available specialized instruments (*e.g.*, mass spectrometers) in the accomplishment of routine analytical determinations." With the advent of the C_2H_2–C_2H_4 assay procedure, these statements are no longer relevant. The C_2H_2–C_2H_4 assay is as crucial for the optimization of biological nitrogen fixation as soil analyses were fundamental to the development of agricultural fertilizer use.

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