*IN SITU STUDIES ON N_2 FIXATION USING THE ACETYLENE REDUCTION TECHNIQUE**

By W. D. P. Stewart,† G. P. Fitzgerald, and R. H. Burris

DEPARTMENT OF BIOCHEMISTRY AND WATER CHEMISTRY LABORATORY, UNIVERSITY OF WISCONSIN, MADISON

Read before the Academy October 24, 1967

The measurement of *in situ* N_2-fixation rates on the basis of total nitrogen changes or $^{15}N_2$ uptake is not entirely satisfactory—the former method is insufficiently sensitive and accurate, and the ^{15}N method is time consuming, expensive, and requires a mass spectrometer. The discovery in 1966 by Schöllhorn and Burris[1] and by Dilworth[2] that the nitrogen-fixing complex (nitrogenase) reduces acetylene to ethylene[2] suggested that the rate of acetylene reduction may be used as an index of the rate of N_2 fixation. Subsequently, the measurement of ethylene production from acetylene[3–7] and the measurement of cyanide,[8] isocyanide,[9] and azide reduction[8, 10] have been used to aid in laboratory studies of N_2 fixation. However, the potential of the method for field investigations of N_2 fixation generally has not been appreciated by limnologists, marine biologists, and soil scientists. In this paper, data obtained in experiments designed to test the feasibility of employing a simple method for measuring acetylene reduction as an index of N_2 fixation in the field illustrate that the method is practical and extremely sensitive.

Sites Studied.—Lake studies were performed in Lake Mendota, Madison, Wisconsin, either offshore or in mid-lake; algal samples also were obtained from Lakes Monona and Wingra. Soil studies were made in the Madison area, studies on nonlegumes on land surrounding Plummer Lake, Vilas County, Wisconsin, and studies on soybeans at the University of Wisconsin experimental farm, Arlington, Wisconsin.

Gases.—Acetylene (purified grade) and a gas mixture of O_2 (22%), CO_2 (0.04%), and argon (78%, high purity) were obtained commercially (Matheson Co.).

Field Method.—Experiments were carried out in 5.0-ml capacity glass serum bottles fitted with rubber serum stoppers. Lake samples (1.0 ml with or without prior concentration, depending on the experiment and the density of the algal population) were added to each bottle. Soil samples 0.78-cm in area and 1-cm deep were taken with a cork borer. Root nodules were detached immediately after the plants were dug, and 100–500 mg fresh weight of nodules was added per bottle. With lake samples, air was removed by flushing the liquid with the premixed gas phase for 1.5 min; then each bottle was stoppered and flushed for a further 1.5 min by introducing gas through a no. 22 hypodermic needle and venting it through a second needle. With soil and with root nodule samples, air was removed from the stoppered bottles through a hypodermic needle with a hand vacuum pump prior to flushing with the premixed gas phase. Evacuation and flushing were performed twice. Acetylene (0.5 ml) or $^{15}N_2$ (1.0 ml) was then injected from a hypodermic syringe, and the samples were incubated *in situ* for the desired period. Generally, 18 samples could be prepared, gassed, and replaced *in situ* for incubation within 30 min of sampling the material. Reactions were terminated by the injection of 50% trichloroacetic acid (0.2 ml to lake water samples and 0.5 or 1.0 ml to root nodule and soil samples). A covering of RTV sealant (General Electric Co.) was applied to each stopper after gassing and after injection of trichloroacetic acid. Samples were returned to the laboratory and analyzed for total nitrogen, for ethylene production, or for ^{15}N enrichment as required.

Laboratory Studies.—Samples of lake algae were exposed in the laboratory using the methods described above or as detailed later. During incubation, the samples were continuously shaken at 30° in a water bath under a constant light intensity of 320 ft-c.

Analysis.—Ethylene formation was detected by gas chromatography with a Varian-Aerograph model 600D gas chromatographic apparatus (H-flame ionization detector) fitted with a 9-ft long,

1/8-in. diameter column containing Porapak R, and run at room temperature. High purity N_2, at a flow rate of about 25 ml/min served as carrier gas. ^{15}N uptake by the test samples was determined by mass spectrometry after measurement of total nitrogen by Kjeldahl digestion, distillation, and nesslerization.[11] Protein values were obtained as total $N \times 6.25$. Control samples of unexposed material, or Millipore (grade HA)-filtered lake water, or of samples to which trichloroacetic acid was added prior to the introduction of acetylene, were always included.

TABLE 1

REDUCTION OF ACETYLENE TO ETHYLENE BY COMMON LAKE ALGAE

Class and genus	Date	Protein/sample (mg)	Mμmoles C_2H_4/mg protein/min
Chlorophyceae			
Cladophora sp.	6/8/67	7.0	0.00
Hydrodictyon sp.	7/14/67	4.9	0.00
Ulothrix sp.	7/6/67	4.1	0.00
Euglenophyceae			
Euglena	7/14/67	2.6	0.00
Myxophyceae			
Anabaena sp.	7/10/67	0.8	0.10
Aphanizomenon flos-aquae	7/10/67	1.8	0.77
Calothrix sp.	6/8/67	7.9	0.01
Gloeotrichia echinulata	7/14/67	1.3	0.36
Lyngbya birgei	8/28/67	1.4	0.00
Microcystis aeruginosa	7/10/67	2.3	0.00
Nostoc sp.	7/14/67	0.9	1.62
Oscillatoria sp.	7/6/67	2.3	0.00

All samples were collected from Lake Mendota except *Anabaena* (Lake Wingra) and *Aphanizomenon* (Lake Monona). Algae were separated when necessary by repeated transfers through Millipore (grade HA)-filtered lake water, and were more than 99% unialgal except for *Calothrix*, which could not be separated from associated nonheterocystous algae. The Lake Wingra *Anabaena* was pale green and unhealthy in appearance—a characteristic feature of blue-green algae from this lake. Exposure period in the laboratory was 30 min, samples being incubated within 30–60 min of collection. Initial gas phase was: C_2H_2 (0.1 atm); O_2 (0.05–0.2 atm); CO_2 (0.0004 atm) or as 0.1 gm $NaHCO_3$/l; argon to 1.0 atm.

Lake Studies.—Various lake algae, including dominant bloom-formers, were obtained as unialgal samples and tested initially for acetylene reduction in the laboratory. The data in Table 1 show clearly that acetylene reduction is associated with blue-green algae of the families Nostocaceae (*Anabaena*, *Aphanizomenon*, and *Nostoc*) and Rivulariaceae (*Calothrix* and *Gloeotrichia*). None of the other samples reduced acetylene. The inability of some samples to reduce acetylene suggests that nitrogen fixation by bacteria associated with the algae is unimportant.

Reproducibility of the *in situ* method was checked and, as Table 2 shows, consistent data were obtained with replicate samples of an unconcentrated algal bloom. Acetylene reduction occurred linearly in the test system employed over a 60-minute period, so that reduction rates are readily obtained from experiments run for periods up to one hour. Data obtained on *in situ* reduction rates over daily and weekly time periods are presented in Figures 1 and 2. Daily variation in acetylene reduction was marked and sometimes rapid (Fig. 1*A*), and was related directly to the abundance of *Gloeotrichia* but not to *Lyngbya* (Fig. 1*B*, Fig. 2); the variation showed only a

TABLE 2

ETHYLENE PRODUCTION *in situ* BY REPLICATE SAMPLES OF BLOOM ALGAE

Sample	Protein (mg)	Mμmoles C_2H_4/mg protein/min
1	0.97	1.75
2	1.04	1.86
3	1.16	1.58
4	1.19	1.62
5	1.19	1.32
6	1.04	1.79
7	1.03	1.49
8	0.93	2.08
9	1.05	1.52
10	1.19	1.74

Samples of 1.0 ml of unconcentrated heavy *Gloeotrichia echinulata* bloom incubated offshore in Lake Mendota on July 27 between 1:40 and 2:10 P.M.

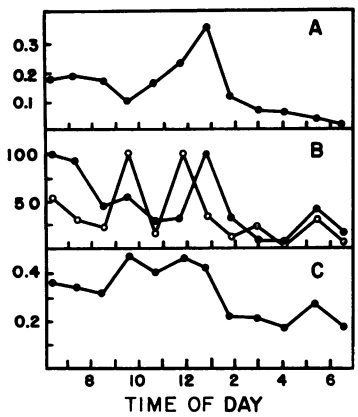

FIG. 1.—Hourly variation in *in situ* acetylene reduction (*A*), abundance of dominant algae (*B*), and total protein (*C*) in the surface waters of Lake Mendota during a heavy algal bloom in July 1967. Units on *y* axis of figure are as follows: (*A*) mμmoles C_2H_2 reduced/1.0-ml sample/min; (*B*) relative abundance of *Gloeotrichia echinulata* (●—●) and *Lyngbya birgei* (○—○)/sample; (*C*) total protein (mg/1.0-ml sample). Incubation period 30 min. Each point is the mean of duplicate determinations. All times in this paper are given as central standard time.

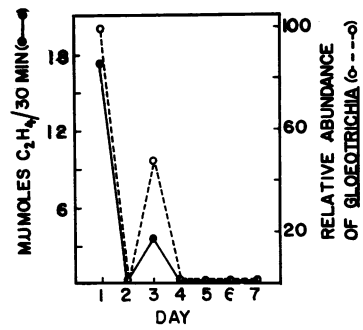

FIG. 2.—Daily variation in *in situ* acetylene reduction and its relationship to the abundance of the dominant bloom-forming alga *Gloeotrichia echinulata* in Lake Mendota during the period August 15–21, 1967. All samples were incubated during the period 11:00 A.M.–1:00 P.M. for 30 min.

general relationship to the total protein per sample (Fig. 1*C*). The falling-off in the reduction rate in Figure 1*A* in midafternoon reflects a decrease in the population of algae in surface waters, probably as a result of heavy rainfall, decrease in light intensity, and a change in wind direction on the day of sampling. Figure 2 illustrates the marked variation in acetylene reduction from day to day as algal blooms developed and disappeared.

The effect of light on reduction was checked using natural lake samples in the field and in the laboratory. As Table 3 shows, reduction *in situ* fell off rapidly when the samples were incubated in the dark; the fall-off rate depends upon the sampling time, being high in early morning and evening and lower at midday. Recovery of light-starved samples was rapid (Fig. 3).

Confirmatory data that the algae are responsible for the light-dependent *in situ*

TABLE 3

VARIATION IN ETHYLENE PRODUCTION BY LAKE SAMPLES INCUBATED IN THE LIGHT AND IN THE DARK

Min of pretreatment	Time at start:	Mμmoles C_2H_4/mg Protein/30 Min					
		5:30 A.M.		11:00 A.M.		5:00 P.M.	
		Light	Dark	Light	Dark	Light	Dark
0		1.6	0.4	1.8	0.9	1.2	0.2
15		1.8	0.6	2.1	0.8	1.0	0.0
30		2.0	0.2	1.6	0.7	0.9	0.0
60		1.6	0.0	1.8	0.1	0.4	0.0
90		1.8	0.0	1.7	0.0	0.0	0.0

Samples of 6.0 liters of Lake Mendota algae were concentrated 100 times with a fine plankton net in August 1967. *Gloeotrichia echinulata*, *Coelosphaerium* sp., and *Lyngbya birgei* were dominant. Duplicate 1.0-ml samples were set up in the dark (aluminum foil) or in the light *in situ*. After the pretreatment period shown above, the samples were further incubated for 30 min with C_2H_2. Sunrise, 5:10 A.M.; marked decrease in light from 4:00 P.M.; darkness, 6:45 P.M. Temperature *in situ*: 5:30 A.M. (21°), 11:00 A.M. (23°), 5:00 P.M. (18°). Protein/sample: 5:30 A.M., 0.71 mg; 11:00 A.M., 0.76 mg; 5:00 P.M., 0.61 mg.

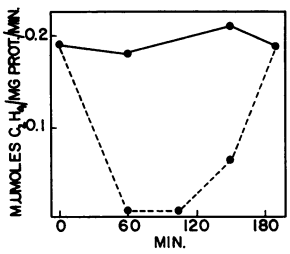

FIG. 3.—Comparison of acetylene reduction by natural populations of Lake Mendota algae incubated in the laboratory in the light and in the dark. Algae were concentrated with a fine plankton net. Dominant algae were *Gloeotrichia echinulata*, *Lyngbya birgei*, and *Aphanizomenon flos-aquae*. Samples initially were exposed to air, and then were exposed to C_2H_2 for 30 min at the times indicated in the figure. ●—● Denotes samples continuously incubated in the light; ○—○ denotes samples incubated in the dark (bottles covered with aluminum foil), and then placed in the light after 105 min. Each point is the mean of triplicate determinations.

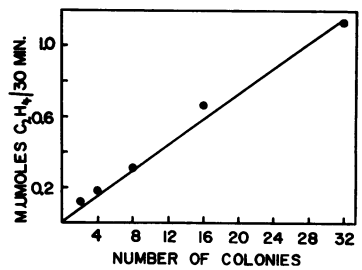

FIG. 4.—Acetylene reduction in relation to the number of *Gloeotrichia echinulata* colonies per 1.0-ml sample. Samples were incubated *in situ* in Lake Mendota in July 1967 for 30 min. Each point is the mean of duplicate determinations.

reduction were obtained in two ways: First, the concentrations of *Gloeotrichia*, the dominant heterocystous bloom-forming alga, in a unit volume of water were varied, and it was found (Fig. 4) that there was a direct relationship in the samples between the abundance of *Gloeotrichia* and the rate of acetylene reduction. Such data also demonstrate the sensitivity of the method—ethylene production can be detected in 30 minutes when 2–4 active *Gloeotrichia* colonies are present per sample. Second, acetylene reduction in Lake Mendota was checked *in situ* at depths down to 18 meters and related to algal abundance. As Figure 5 shows, total algal biomass and acetylene reduction were greatest in the surface 3 meters. Reduction rates were directly related to the abundance of heterocystous algae which showed a distinct stratification in the top 3 meters, with *Gloeotrichia* dominant in the 0- to 2-meter layer and *Anabaena* restricted to the 3-meter zone.

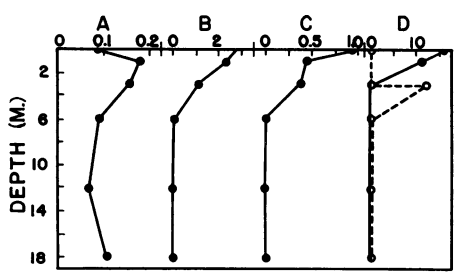

FIG. 5.—Acetylene reduction *in situ* at various depths in Lake Mendota in July 1967 in relation to total protein and algal abundance. Units on x axis of figure are as follows: (*A*) mg protein/1.0-ml sample; (*B*) mμmoles C_2H_2 reduced/1.0-ml sample/30 min; (*C*) mμmoles C_2H_2 reduced/1.0 mg N/min; (*D*) number of units of *Gloeotrichia echinulata* (●—●) and *Anabaena* sp. (○—○) per 1.0-ml sample. Each point is the mean of duplicate determinations.

Soil Studies.—The data obtained by taking replicate samples of known area and depth from three soils and analyzing these for a capacity to reduce acetylene are shown in Table 4. Ethylene production occurred rapidly in a soil rich in blue-green algae (soil 1), was absent in samples of an intermediate soil with no observable blue-green algae and only a light grass cover (soil 2), and occurred slowly in a well-established turf (soil 3). In the soil rich in algae, acetylene reduction was characteristic of the surface centimeter only, and, unlike the well-established turf,

TABLE 4

ACETYLENE REDUCTION BY SOIL SAMPLES INCUBATED *in situ*

Sample	Mμmoles C_2H_4/Sample/30 Min Soil 1	Soil 2	Soil 3
1	4.4	0.0	0.3
2	6.6	0.0	0.3
3	6.0	0.0	0.2
4	4.7	0.0	0.5
5	3.9	0.0	0.3
6	3.7	0.0	lost

See text for a description of the soils. The mean *N* (mg/sample) and temperature were: soil 1 (4.3 and 32°); soil 2 (4.1 and 32°); soil 3 (2.4 and 39°). Incubation period was 30 min on August 3, 1967. Temperature inside the bottles did not vary from soil temperature by more than 0.2° during incubation period.

TABLE 5

EFFECT OF LIGHT/DARK REGIME AND EXOGENOUS CARBOHYDRATE ON ACETYLENE REDUCTION IN SOILS

	Time (min)	—Mμmoles C_2H_4/Sample/30 Min— Light	Light + 2% sucrose	Dark
Soil 1	0	16.1	–	—
	90	12.5	14.3	7.5
	210	8.8	5.3	0.8
Soil 3	0	0.4	–	—
	90	0.4	0.4	0.4
	210	0.3	0.2	0.5

Soils are described in the text. Mean *N* (mg) per sample: soil 1 (4.9); soil 3 (1.8). All samples were treated at 0 time (0.5 ml sucrose solution added per sample; dark samples were covered with aluminum foil). Samples were exposed to C_2H_2 for 30 min in light or dark at times from start indicated. Experiment began at 1:00 P.M. on August 4, 1967. Temperature of soils 1 and 3 was, respectively: 0 min, 34°, 38°; 90 min, 31°, 33°; 210 min, 30°, 30°.

was largely light-dependent (Table 5). Such data are similar to those obtained previously using ^{15}N in sand-dune slack soils.[12] Acetylene reduction was not stimulated over a four-hour period by exogenous carbohydrate (Table 5). Algae thus seem responsible for the observed reduction in soil 1, as acetylene reduction decreased much more rapidly in the dark than in the light. The heterotrophic organisms may be primarily responsible for reduction in soil 3, in which acetylene reduction was maintained in the dark. Heterotrophic reduction did not respond to added carbohydrate during the period tested.

Root Nodule Studies.—Data on acetylene reduction by nodules, roots, and soil associated with *Alnus* and *Comptonia* root systems are presented in Table 6. The capacity to reduce acetylene is characteristic of root nodules, either detached or attached to portions of the root. Associated soil samples or nodule-free roots did not reduce appreciable quantities of acetylene, indicating that free-living nitrogen-fixing microorganisms were unimportant in these soils. As Figures 6–8 show, ethylene production proceeded linearly over a 60-minute period. The time course data for *Comptonia* (Fig. 6) are interesting in that ethylene production by nodules sampled in the morning was only about half that by nodules sampled in the afternoon. This implies that acetylene reduction, like N_2 fixation, depends upon the availability of photosynthetic products from the higher plant.

TABLE 6

ACETYLENE REDUCTION BY NODULES, ROOTS, AND SOIL ASSOCIATED WITH *Alnus rugosa* AND *Comptonia peregrina*

		Roots	Nodules	Nodulated roots	Soil
Alnus	N (mg)	0.8	2.4	—	1.4
	C_2H_4 (mμmoles/bottle)	0.3	331	247	0.0
	C_2H_4/mg *N*/min	0.01	4.58	–	0.00
Comptonia (Expt. 1)	N (mg)	1.3	1.4	—	4.8
	C_2H_4 (mμmoles/bottle)	0.0	52	240	0.2
	C_2H_4/mg N/min	0.00	1.25	—	0.01
Comptonia (Expt. 2)	N (mg)	4.9	1.8	—	1.7
	C_2H_4 (mμmoles/bottle)	0.0	48	12	0.0
	C_2H_4/mg N/min	0.00	0.9	—	0.00

The exposure period was 30 min on August 6 and 7, 1967. The time and temperature at the start were: *Alnus* (8:00 A.M., 17.5°), *Comptonia* 1 (3:00 P.M., 18.5°), *Comptonia* 2 (5:05 P.M., 17.0°). Incubations were started within 30 min of sampling nodules. Each value is the mean of duplicate (*Comptonia* 1) or triplicate determinations.

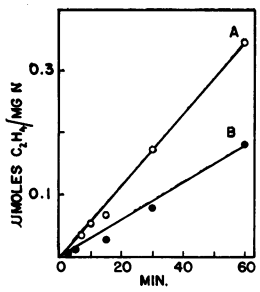

FIG. 6.—Time course of acetylene reduction by *Comptonia peregrina* root nodules in August 1967. Series (*A*) nodules were sampled from plants dug at 1:30 P.M.; series (*B*) nodules were sampled from plants dug at 7:30 A.M. Mean temperature: (*A*) (18.5°), (*B*) (17.5°). Each point is the mean of triplicate determinations. ^{15}N enrichment of comparable samples exposed to 70 atom per cent $^{15}N_2$ for 60 min was: (*A*), 0.118; and (*B*), 0.102 atom per cent excess ^{15}N.

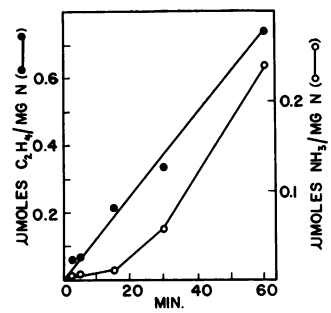

FIG. 7.—Time course of acetylene reduction and N_2 reduction by *Alnus rugosa* root nodules in August 1967. Nodules were sampled from plants dug between 11:00 and 11:30 A.M. Mean temperature was 19°. ^{15}N content of gas phase was 70 atom per cent. Each point is the mean of triplicate determinations.

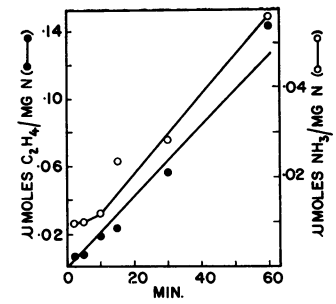

FIG. 8.—Time course of acetylene reduction and N_2 reduction by soybean root nodules in August 1967. Nodules were sampled from plants dug at 9:45 A.M. Mean temperature was 26.5°. ^{15}N content of gas phase was 59 atom per cent. Each point is the mean of triplicate determinations.

With *Alnus* (Fig. 7) and soybean (Fig. 8), ethylene production proceeded linearly for 60 minutes, but fixation of $^{15}N_2$ showed an initial lag. The initial differences in the rates of acetylene and nitrogen reduction may reflect differences in the rates of penetration of these gases to the N_2-fixing sites of the nodules, for the *Alnus* nodules had a typical thick cork covering, and the soybean nodules were large (up to 0.75 cm in diam.). The relatively high concentration of ^{15}N in the 2.5 and 5 minute ^{15}N samples of Figure 8 might be interpreted as reflecting a slow inactivation of the nodules by trichloracetic acid, but this seems unlikely because acetylene reduction was linear with time when the same inactivation method was used. It is more probable that the high values are not significant but arose because of the high relative errors inherent in analysis of samples with such low enrichment with ^{15}N (0.009–0.010 atom per cent ^{15}N excess).

The rates of ethylene production (μmoles/gm fresh nodules/hr) were rather similar to those obtained in laboratory experiments[3, 6] and were: *Comptonia* (0.7–2.2), *Alnus* (4.5), and soybean (1.9).

Discussion.—Studies of cell-free extracts from various N_2-fixing systems have shown that the reduction of acetylene to ethylene is catalyzed by the N_2-fixing complex (nitrogenase). The feasibility of using the rate of ethylene production as an index of N_2-fixation *in situ* is clearly demonstrated by the present study. The method has numerous advantages over the conventional ^{15}N technique which has been used previously in studies on *in situ* N_2 fixation.[13–15] Three points in particular recommend the method. First, it is relatively inexpensive, as the only major piece of equipment required is the gas chromatographic apparatus which costs approximately a tenth as much as a mass spectrometer; ancillary equipment is very

simple. Purified acetylene costs about a cent a liter and 95 atom per cent excess $^{15}N_2$ costs over 600 dollars a liter. Second, the sensitivity of the technique is such that we have measured ethylene production by 0.5-gm quantities of fresh soybean nodules after as little as a five-second exposure to acetylene. In studies on algae, a 30-minute exposure period has been satisfactory. These short incubation times minimize changes which may occur inside the reaction vessels compared with outside conditions. Furthermore, it makes measurements of short-term changes feasible (for example, the hourly variations in N_2 fixation in Lake Mendota). In most investigations using $^{15}N_2$, exposure periods of 6–24 hour have been employed, and although such long exposures have not always been justified, nevertheless the relatively low sensitivity of the ^{15}N-method requires substantially longer sample exposures than the acetylene method. Third, the equipment is easy to handle. This stems largely from the fact that the N_2-fixing complex has a higher affinity for acetylene than for N_2 at a comparable partial pressure of the gasses.[5] Thus, air contamination is not as serious as when $^{15}N_2$ is employed, and somewhat simpler equipment can be used. Also, gas chromatography is a much less demanding and a more rapid technique to master and to apply than mass spectrometry. On the basis of these factors alone, the method is clearly more practical than the ^{15}N technique for the survey of N_2 fixation in the field.

In our lake and soil studies, acetylene reduction occurred consistently and at appreciable rates only when heterocystous blue-green algae were present. This agrees with previous ^{15}N data, for *Nostoc* is a well-known N_2-fixer,[16] *Gloeotrichia* has been shown to fix N_2 in unialgal cultures,[17] and *Aphanizomenon*, the only heterocystous alga reported not to reduce N_2,[17] reduces acetylene. A further check on *Aphanizomenon* using ^{15}N is desirable. The lake studies show that the abundance and physiological activity of the algae vary markedly at different places and at different times (see, for example Figs. 1, 2, 5). Thus, the N_2-fixing activity of lake algae cannot be regarded as uniform even over short periods; rather, the system is in constant flux. This necessitates much more extensive studies on N_2 fixation *in situ* than have previously been feasible with the relatively expensive and time-consuming ^{15}N method. The acetylene-reduction technique provides a simple method with which this can be achieved.

The linearity of the time course for ethylene production from acetylene in root nodules simplifies quantitation of the data on the effectiveness of the nodules in N_2 fixation. Such data have applications in agricultural practice and in studies on the nitrogen cycle in natural ecosystems. The rate of acetylene reduction by root nodules is somewhat greater than by natural populations of algae per unit of nitrogen present. Thus, when nodulated plants and blue-green algae both are abundant, the nodulated plants probably are more important.

The data for *Comptonia* show that the root nodules, which arise on fine lateral roots and which bear numerous nodule rootlets, reduce acetylene and fix N_2. The data obtained with as little as 2.5 minutes of exposure to acetylene confirm previous data obtained by Ziegler and Hüser[18] who employed $^{15}N_2$ and a 6-day incubation period.

Reduction of N_2 to 2 NH_3 requires the transfer of six electrons, whereas the reduction of acetylene to ethylene requires two electrons. On this basis, it might be anticipated that the reduction of acetylene to ethylene should occur three times as rapidly as the reduction of N_2 to 2 NH_3 (molar ratio ethylene: NH_3 equals 1.5).

The correlation between the rates of acetylene reduction and N_2 fixation has not been studied extensively in these experiments. However, Schöllhorn and Burris[5] reported a value near 1.25 for *Clostridium* and *Azotobacter*, and Klucas[19] found an average ratio of 1.59 for ethylene formed to NH_3 formed with purified extracts from *A. vinelandii*. More data are required to establish how variable the ratio is in N_2-fixing systems, as this ratio is necessary to convert estimates of acetylene reduction to N_2 fixation in the field.

Because the reduction of acetylene is an indirect index of N_2 fixation, we urge investigators to verify their critical observations on acetylene reduction by demonstrating fixation of $^{15}N_2$ under the same conditions. This is merely an admonition to refer routinely to a primary standard rather than to rely entirely upon an indirect secondary standard, attractive though it may be. In this regard, it is our opinion that acetylene reduction furnishes a more reliable index of N_2 fixation than reduction of cyanide, isocyanide, or azide. This opinion cannot be convincingly documented at present, but inhibitory effects by cyanide, isocyanide, and azide and differential inactivation of N_2 fixation versus reduction of these compounds raise doubts regarding their reliability as an index of N_2 fixation.

Summary.—The reduction of acetylene can be employed as an index of N_2-fixation *in situ*, in aquatic environments, in soils, and by nodulated plants. Ethylene produced from acetylene could be measured gas-chromatographically after 5 seconds to 30 minutes of exposure of N_2-fixing agents to acetylene. In lakes and soils, reduction was correlated directly with the abundance of heterocystous blue-green algae. Acetylene reduction by *Alnus*, *Comptonia*, and soybean was characteristic of the root nodules only.

* Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. This investigation was supported in part by grant GB-483 from the National Science Foundation and by grant AI-00848 from the National Institutes of Health. We wish to thank Dr. C. A. Kust for supplying the soybean plants used in our experiments.

† Present address: Department of Botany, Westfield College, University of London, London, N.W.3, England.

[1] Schöllhorn, R., and R. H. Burris, *Federation Proc.*, **25**, 710 (1966).
[2] Dilworth, M. J., *Biochim. Biophys. Acta*, **127**, 285 (1966).
[3] Koch, B., and H. J. Evans, *Plant Physiol.*, **41**, 1748 (1966).
[4] Koch, B., H. J. Evans, and S. Russell, *Plant Physiol.*, **42**, 466 (1967).
[5] Schöllhorn, R., and R. H. Burris, these PROCEEDINGS, **58**, 213 (1967).
[6] Sloger, C., and W. S. Silver, *Bacteriol. Proc.*, p. 112 (1967).
[7] Kelly, M., R. V. Klucas, and R. H. Burris, *Biochem. J.*, **105**, 3c (1967).
[8] Hardy, R. W. F., and E. Knight, Jr., *Biochim. Biophys. Acta*, **139**, 69 (1967).
[9] Kelly, M., J. R. Postgate, and R. L. Richards, *Biochem. J.*, **102**, 1C (1967).
[10] Schöllhorn, R., and R. H. Burris, these PROCEEDINGS, **57**, 1317 (1967).
[11] Burris, R. H., and P. W. Wilson, in *Methods in Enzymology*, ed. S. P. Colowick and N. O. Kaplan (New York: Academic Press, 1957), vol. 4, p. 355.
[12] Stewart, W. D. P., *Ann. Botany*, **29**, 229 (1965).
[13] *Ibid.*, **31**, 385 (1967).
[14] Neess, J. C., R. C. Dugdale, V. A. Dugdale, and J. J. Goering, *Limnol. Oceanog.*, **7**, 163 (1962).
[15] Bond, G., *New Phytologist*, **55**, 147 (1956).
[16] Stewart, W. D. P., *Nitrogen Fixation in Plants* (London: Athlone Press of the University of London, 1966).
[17] Williams, A. E., and R. H. Burris, *Am. J. Botany*, **39**, 340 (1952).
[18] Ziegler, H., and R. Hüser, *Nature (London)*, **199**, 508 (1963).
[19] Klucas, R. V., Ph.D. thesis, University of Wisconsin (1967).