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The process of biological nitrogen fixation involves the enzymic reduction of atmospheric nitrogen (N_2) to ammonia (NH_3). The enzyme which catalyzes this reduction, nitrogenase, does not exhibit a high degree of substrate specificity. It also catalyzes the reduction of acetylene (C_2H_2) to ethylene (C_2H_4). The reduction of C_2H_2 to C_2H_4 coupled with gas chromatography has been widely used as a method of measuring nitrogenase activity.

In this investigation, the acetylene reduction assay was adapted for the measurement of nitrogenase activity of intact nodulated cultures of legumes. An advantage of the method is that cultures may be assayed for nitrogenase activity in situ without serious damage to the plants. Entire cultures were placed in polyethylene wastebaskets and then were exposed to acetylene after sealing the wastebaskets with plastic lids. Time course experiments showed that rates of acetylene

reduction were linear for periods of at least two hours. The correlation coefficient between rates of acetylene reduction and nodule fresh weight was 0.79. Acetylene reduction rates of either intact nodulated plants in Perlite or nodulated root systems removed from the Perlite were approximately 40% greater than acetylene reduction rates of detached nodules from comparable cultures. No consistent diurnal variation was observed in nitrogenase activity of nodulated plants grown under controlled environmental conditions. The method is useful for the assessment of nitrogenase activity of legume cultures in a porous medium under standardized conditions, but its application to legumes in soil is complicated by factors, such as soil moisture content, that influence rates of gaseous diffusion.

Measurement of Nitrogenase Activity of Intact Legume Symbionts *in situ* Using the Acetylene Reduction Assay

by

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MEASUREMENT OF NITROGENASE ACTIVITY OF INTACT LEGUME SYMBIONTS IN SITU USING THE ACETYLENE REDUCTION ASSAY

INTRODUCTION

Nitrogen is an essential element for all living organisms and is exceeded in concentration in most living tissues only by carbon, hydrogen and oxygen. As the diatomic gas N_2, nitrogen is quite abundant in the earth's atmosphere, accounting for approximately 78% by volume of the composition of dry air. However, all animals and the large majority of plants are unable to utilize the N_2 of the atmosphere directly. Direct utilization of N_2 is restricted to a relatively small number of microorganisms which, either individually or in symbiotic association with a higher plant, possess the capacity to enzymically reduce N_2 to ammonia (NH_3). The latter process is termed "biological nitrogen fixation." While restricted to a relatively few species, the magnitude of biological nitrogen fixation is astounding as evidenced by Donald's (1960) estimate that 10^8 tons of N_2 are biologically reduced to NH_3 annually.

Prominent among the free living microorganisms known to be capable of reducing N_2 to NH_3 are representatives of 11 families of bacteria, including obligate anaerobes such as Clostridium pasteurianum, strict aerobes such as Azotobacter vinelandii and faculative anaerobes including Klebsiella pneumoniae. Representatives of six

families of blue-green algae, most abundantly members of the Nostocaceae, also fix nitrogen. The most important microorganisms fixing nitrogen symbiotically are species of the genus Rhizobium which inhabit legume root nodules. Of the approximately 15,000 species of the Leguminosae, nodules have been detected on the majority of the species examined. Thirteen species of non-leguminous angiosperms which grow symbiotically with microorganisms believed to be actinomycetes also reduce N_2 to NH_3. In addition, certain cycads, aquatic ferns, liverworts and lichens growing with blue-green algae have been reported to fix nitrogen (Nutman, 1971).

Increasing concern over the world food protein deficit (Hardy, 1971a; Nutman, 1971) has stimulated interest in the possibility that many more organisms than presently known are capable of reducing atmospheric nitrogen. With the development of more sensitive, rapid and less expensive methods of detecting nitrogen fixing activity, the probability of recognizing these organisms has been greatly increased.

In recent years, much attention in the study of nitrogen fixation has been directed toward the use of cell-free extracts to elucidate the chemistry and biochemistry of the fixation process. Yet answers to more practical problems such as how to feed the ever-increasing populations of underdeveloped nations must come from investigations of the whole plant. Developments in the genetics, physiology and

ecology of nitrogen fixation are dependent upon methods for measuring the process in intact plants.

Chemically, the reduction of N_2 to NH_3 involves the following reaction:

$$N_2 + 6e^- + 6H^+ \xrightarrow{\text{nitrogenase}} 2NH_3$$

Ammonia is the first detectable stable product. However, investigations on the mechanism of the reaction suggest that the enzyme catalyzes three 2-electron reductions resulting in the production of enzyme-bound intermediates at the oxidation level of diimide (HN = NH) and hydrazine ($H_2N - NH_2$) (Hardy and Knight, 1967). Although nitrogenase is absolutely necessary for the reduction of N_2 to NH_3, the enzyme does not exhibit a high degree of substrate specificity. Substantial evidence has established that the enzyme catalyzes the reduction of a variety of compounds including N_2O, N_3^-, alkynes, HCN, nitriles and isonitriles. Several of these compounds including acetylene were originally observed to be inhibitors of nitrogen fixation; subsequently they have been reclassified as alternate substrates of the enzyme (Hardy and Burns, 1968).

Routine laboratory methods for measuring nitrogen fixation by living organisms, such as Kjeldahl analyses and ^{15}N enrichment by mass spectrometry, are extremely time consuming, expensive and relatively insensitive. Consequently, they are not very amenable to large scale laboratory or field studies. Following the reports of

Dilworth (1966) and Schöllhorn and Burris (1966) that acetylene was an inhibitor of nitrogen fixation and that acetylene was reduced to ethylene by the nitrogen fixing system, Hardy and Knight (1967) proposed the application of the reduction of acetylene to ethylene coupled with gas chromatography as a sensitive assay procedure for detecting and measuring nitrogenase activity. Extensive use of this assay in the laboratory as well as in the field has been made in recent years. Koch and Evans (1966) used the reduction of acetylene to ethylene as a measure of nitrogen fixing activity in laboratory studies on soybean root nodules. Koch and Evans (1967), Hardy et al. (1968), Roughley and Dart (1969), Sprent (1969, 1971a, 1971b), Schwinghamer et al. (1970) and Bergersen (1970) have applied this assay to laboratory investigations on the nitrogenase enzyme and detached legume and non-leguminous root nodules. Field studies by Stewart et al. (1967), Hardy et al. (1968), Silver and Mague (1970) and Mague (1970, 1971) have further confirmed and extended the usefulness of this assay.

Although nitrogen and acetylene are chemically very different, they are structurally isosteric and isoelectronic (Schöllhorn and Burris, 1966, 1967; Dilworth, 1966; Hardy and Knight, 1967). Ethylene appears to be the sole product of acetylene reduction. If other gases such as methane are produced, they are present in concentration $< 0.01\%$ of ethylene (Hardy et al., 1971b). Catalysis of acetylene reduction by nitrogenase has the same requirements for

reductant, Mg^{++}, ATP and anaerobic conditions as does the reduction of nitrogen to ammonia. Dilworth (1966), Schöllhorn and Burris (1967), Hardy et al. (1968) and Klucas et al. (1968) have shown that acetylene reducing activity parallels nitrogen fixing activity during the purification of nitrogenase from free living bacteria and legume nodule bacteroids. Furthermore, the relationship between nitrogenase activity as measured by the acetylene reduction method and rates of nitrogen fixation have already been firmly established (Dilworth, 1966; Schöllhorn and Burris, 1966; Koch and Evans, 1967; Stewart et al., 1967; Hardy et al., 1968; Bergersen, 1970; Akkermans, 1971; Mague, 1971). These facts establish the validity of the acetylene reduction method for detecting and measuring nitrogenase activity.

Conversion of the amounts of acetylene reduced to ethylene to absolute values of nitrogen fixed is based upon the stoichiometric relationship between C_2H_2 reduced and N_2 fixed. While the nitrogenase catalyzed reduction of N_2 to $2NH_3$ requires the transfer of six electrons, the analogous reduction of acetylene to ethylene requires the transfer of only two electrons. Therefore, a theoretical factor (C_2H_2 reduced: N_2 fixed) of three has been used in most conversions. Stewart et al. (1967) obtained a value of 3.9 for detached soybean nodules, while Bergersen (1970) and Mague (1971) obtained values of 2.7 - 4.2 and 1.9, respectively, with soybean nodules. Nitrogen fixation involves the reduction of N_2 to NH_3 and incorporation of NH_3

into protein through a series of complex reactions, but acetylene reduction in the presence of nitrogenase is a simple reduction process which produces a volatile product. It is not surprising, therefore, that the experimentally determined values relating rates of acetylene reduction to rates of nitrogen fixation often deviate somewhat from the expected theoretical value.

This investigation involves the application of the acetylene reduction assay to measuring nitrogenase activity in intact plants. An apparatus has been designed which includes polyethylene incubation chambers capable of accommodating large tissue samples and even whole potted plants. Detailed investigations of various factors and conditions affecting the assay have been conducted. Recommendations are presented which hopefully will be valuable to other investigators applying the assay system to large samples of materials, potted plants and plants removed from native soils.

MATERIALS AND METHODS

Culture Conditions

Soybean (Glycine max var Chippewa) plants inoculated with a commercial strain of Rhizobium japonicum ("S" culture, Nitragin Company) were grown in a greenhouse in eight inch plastic pots containing Perlite under a 16-hour photoperiod (500 foot candles) for approximately two to three weeks. The plants were irrigated daily with a nitrogen-free nutrient solution except on the fourth day when they were irrigated with tap water to prevent salt accumulation. At least two days prior to each experiment, the plants were transferred to a controlled environment chamber, maintained on a 16-hour photoperiod (1000 foot candles) at 24° C and an 8-hour dark period at 18° C. While in the chamber, cultures were irrigated daily with 600 ml of nitrogen-free nutrient solution or with tap water. Pots were thinned to a uniform number of plants (8-10) prior to each experiment.

In experiments in which the plants were grown in soil, five plants, grown as previously described, and at approximately 10 days of age, were transplanted into a pot containing a weighed amount of soil. After 7-10 days for establishment in the soil, the plants were moved to a controlled environment chamber (as above). In studies of the effects of soil moisture content on assaying acetylene reduction

in plants rooted in soil, designated cultures were irrigated at different soil moisture levels for at least three days prior to each experiment.

Assay for Acetylene Reduction

All assays for acetylene reduction were performed with 21 liter polyethylene containers each of which was covered by a Plexiglas lid fitted with a rubber serum stopper. Intact plants, excised roots or detached nodules were placed in the container; the lid was sealed to the container with Vasoline (Pure Petroleum Jelly, Chesebrough-Pond's Inc.) and secured tightly by use of wire "S" hooks and a rubber band (Figure 1). A volume of air equivalent to the volume of acetylene to be injected was removed from the sealed container using a 50-ml hypodermic syringe. Acetylene was either generated by the addition of calcium carbide to distilled water or was obtained from a cylinder of compressed gas. When cylinder gas was utilized, it was purified by passing through H_2SO_4 and H_2O traps.

Various volumes of acetylene were added to the sealed container, using a 50-ml hypodermic syringe, to give the desired partial pressure (p) of acetylene. The air-acetylene mixture was immediately mixed by repeatedly and rapidly filling and emptying a 50-ml hypodermic syringe. This procedure was repeated at 15 minute intervals throughout the incubation period of each experiment and just prior

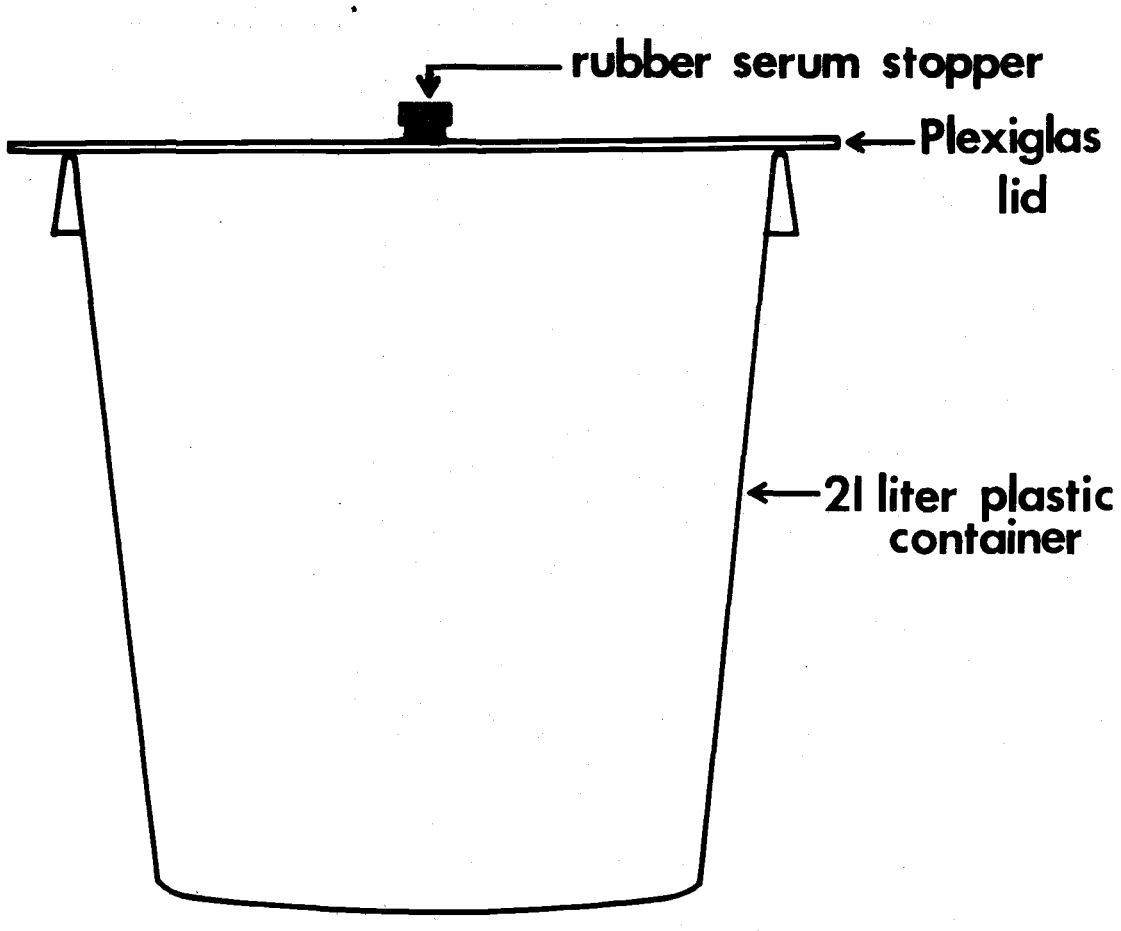

Figure 1. Diagram of the experimental apparatus. Plexiglas lid was sealed to the polyethylene containers with Vasoline and secured tightly by the use of wire "S" hooks and a rubber band.

to sampling. At the conclusion of an incubation period, 1-ml gas samples were withdrawn (by the use of 1-ml hypodermic syringes) from the container and the tip of the needle inserted into a rubber stopper to prevent gas leakage. Alternatively, samples were removed and stored in evacuated blood tubes. A 30-minute incubation at pC_2H_2 0.025 atm (see Results and Discussion) was the standard assay.

Ethylene content was determined using a Varian-Aerograph model 600 D gas chromatograph equipped with a hydrogen flame ionization detector and a Sargent recorder. Nitrogen was the carrier gas at a flow rate of 25 ml/min. The column, 6 feet long by 1/8 inch in diameter, was packed with Poropak R (Type R, 80-100 mesh, 30-10 PAK, Waters Associates, Inc.) and maintained at 50° C. Ethylene determinations were based on the average of at least three 1-ml sample injections. Peak areas for ethylene and acetylene were directly proportional to their respective concentrations over the range utilized in all assays. The retention time for ethylene was approximately 58 seconds and for acetylene approximately 78 seconds.

RESULTS AND DISCUSSION

Establishment of Assay Procedure

Preliminary experiments to establish the validity of the experimental apparatus indicated: 1) the polyethylene container, Plexiglas lid, rubber serum stopper and Vasoline produced no detectable acetylene or ethylene; 2) there was no detectable leakage of acetylene or ethylene from the sealed container; 3) pots containing only Perlite or soil (no plants) irrigated in the same manner as the cultured plants produced no measureable acetylene or ethylene; 4) no measurable quantity of ethylene was produced by non-nodulated plants; and 5) no measurable ethylene was produced by plants incubated in the absence of acetylene. There was no appreciable change in temperature inside the assay chamber during a two-hour assay period.

The kinetics of acetylene reducing activity were observed in a time course experiment (Figure 2). Following an initial lag of < 15 minutes, ethylene production was linear throughout a two-hour assay. The initial lag was nearly eliminated when the pC_2H_2 was increased to 0.05 atm (Figure 3). The lag period apparent in Figure 2 may be a result of slower diffusion of acetylene into and ethylene out of the nodule tissue at the lower acetylene level. Koch and Evans (1966), using excised soybean root nodules, found that ethylene production was linear up to 1-2 hours, while Sprent (1969), also using soybean

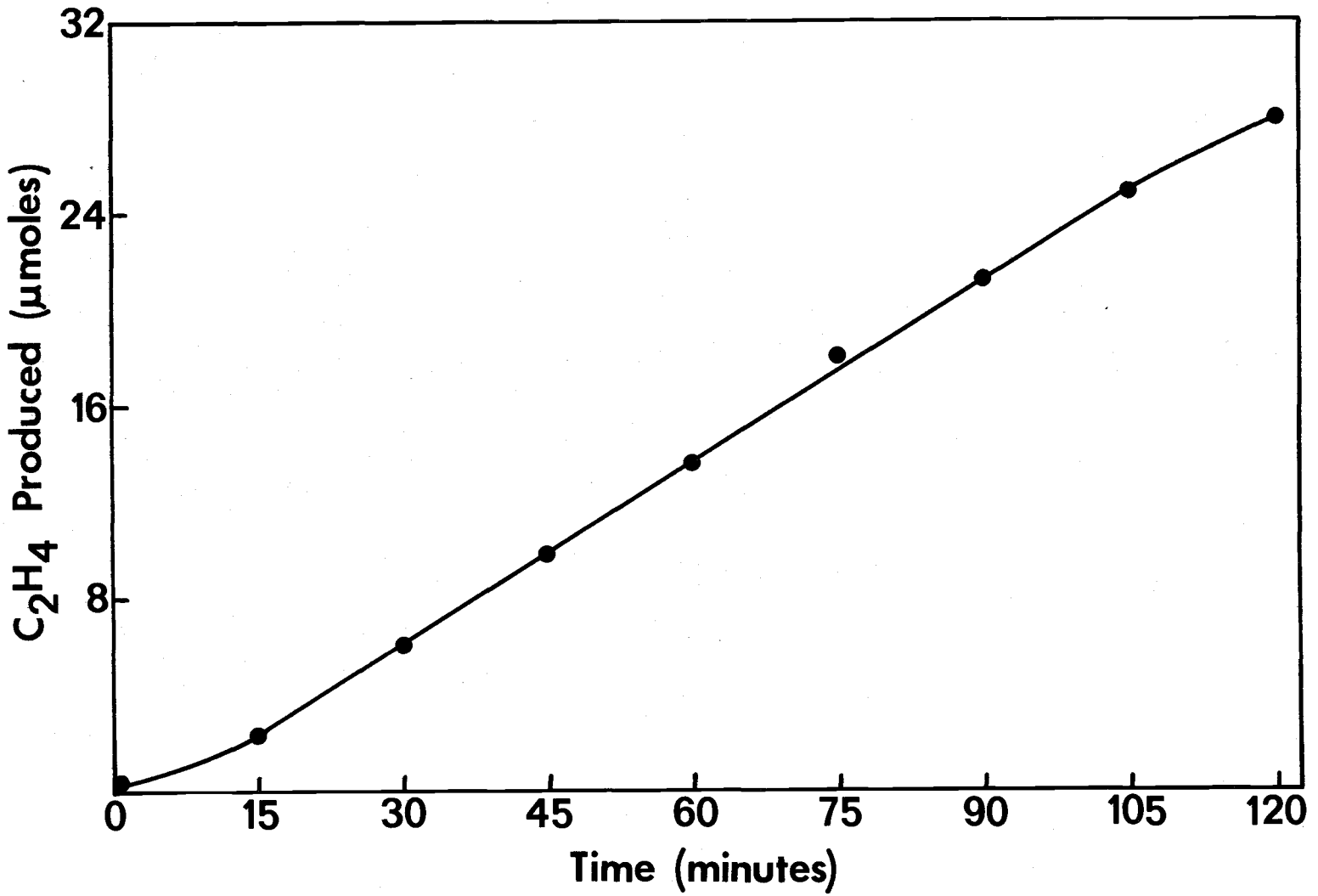

Figure 2. Time course of acetylene reduction by plants growing in Perlite.

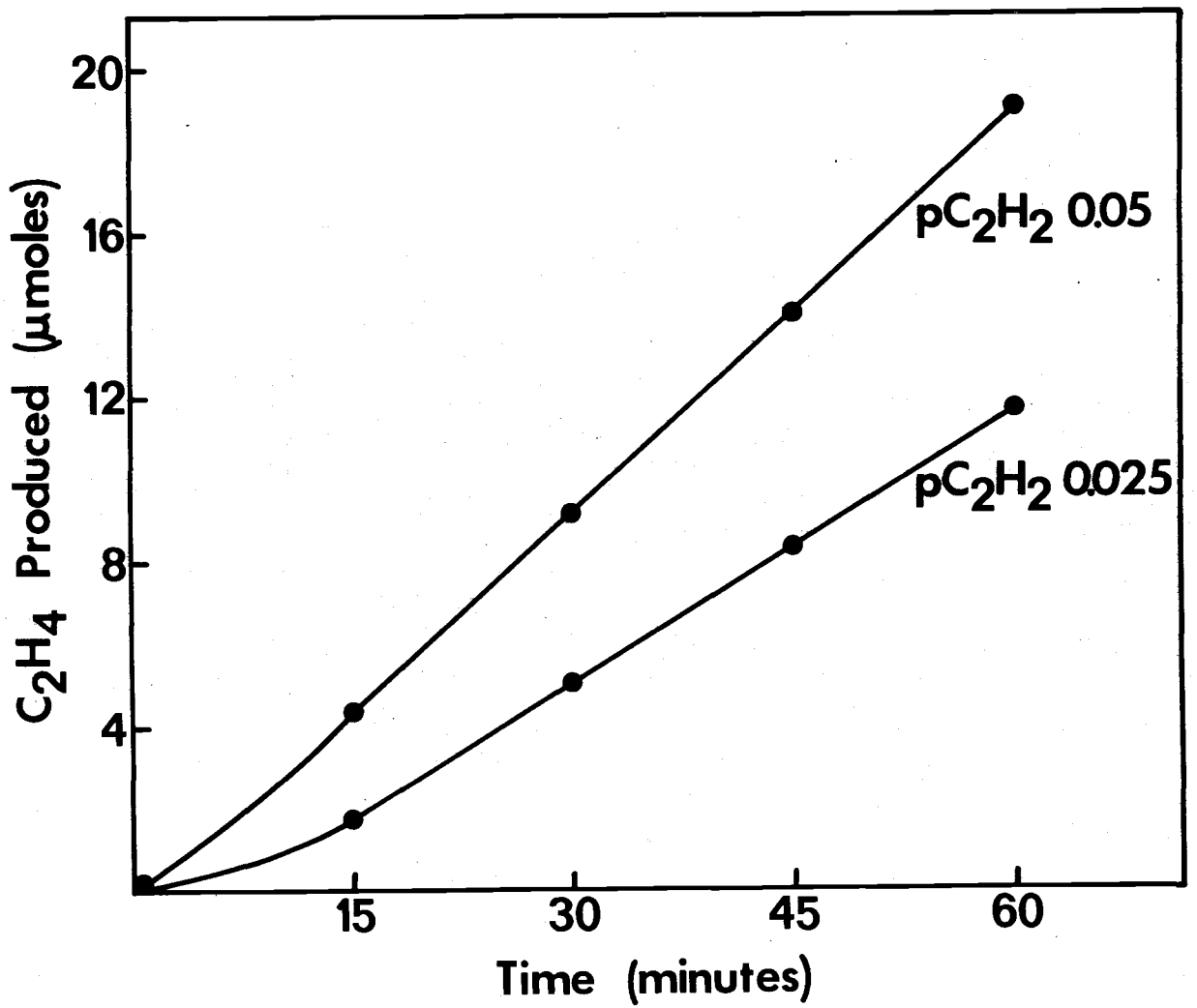

Figure 3. Time course of acetylene reduction by plants growing in Perlite at two C_2H_2 levels.

nodules, observed linearity up to eight hours provided special attention was given to the nodule-gas volume ratio. Using nodulated soybean roots, Stewart et al. (1967) and Hardy et al. (1968) obtained linear kinetics up to 60 minutes.

Saturation of Nitrogenase with Acetylene

Partial pressures up to 0.1 atm C_2H_2 were insufficient to saturate nodule nitrogenase in the culture (Figure 4). At pC_2H_2 levels above 0.05 atm, the experimental apparatus collapsed during the initial evacuation. Since the rate of ethylene production was constant for two hours at pC_2H_2 0.025 atm and the volume of the experimental apparatus was maintained at this level of the gas, subsequent experiments were run for 30 minutes at pC_2H_2 0.025 atm. Failure to obtain enzyme saturation is in contrast to previous investigations, all of which made use of more rigid assay chambers. Koch and Evans (1966) obtained saturation with detached soybean nodules at pC_2H_2 0.10 atm, while Mague (1971) did not observe saturation with soybean nodules until a pC_2H_2 0.20 atm was utilized. Using nodulated soybean root systems, Hardy et al. (1968) established saturation from pC_2H_2 0.025 - 0.20 atm. The apparent inhibition observed by Hardy et al. (1968) and Mague (1971) at high levels of acetylene may actually reflect a lowered pO_2 rather than acetylene toxicity.

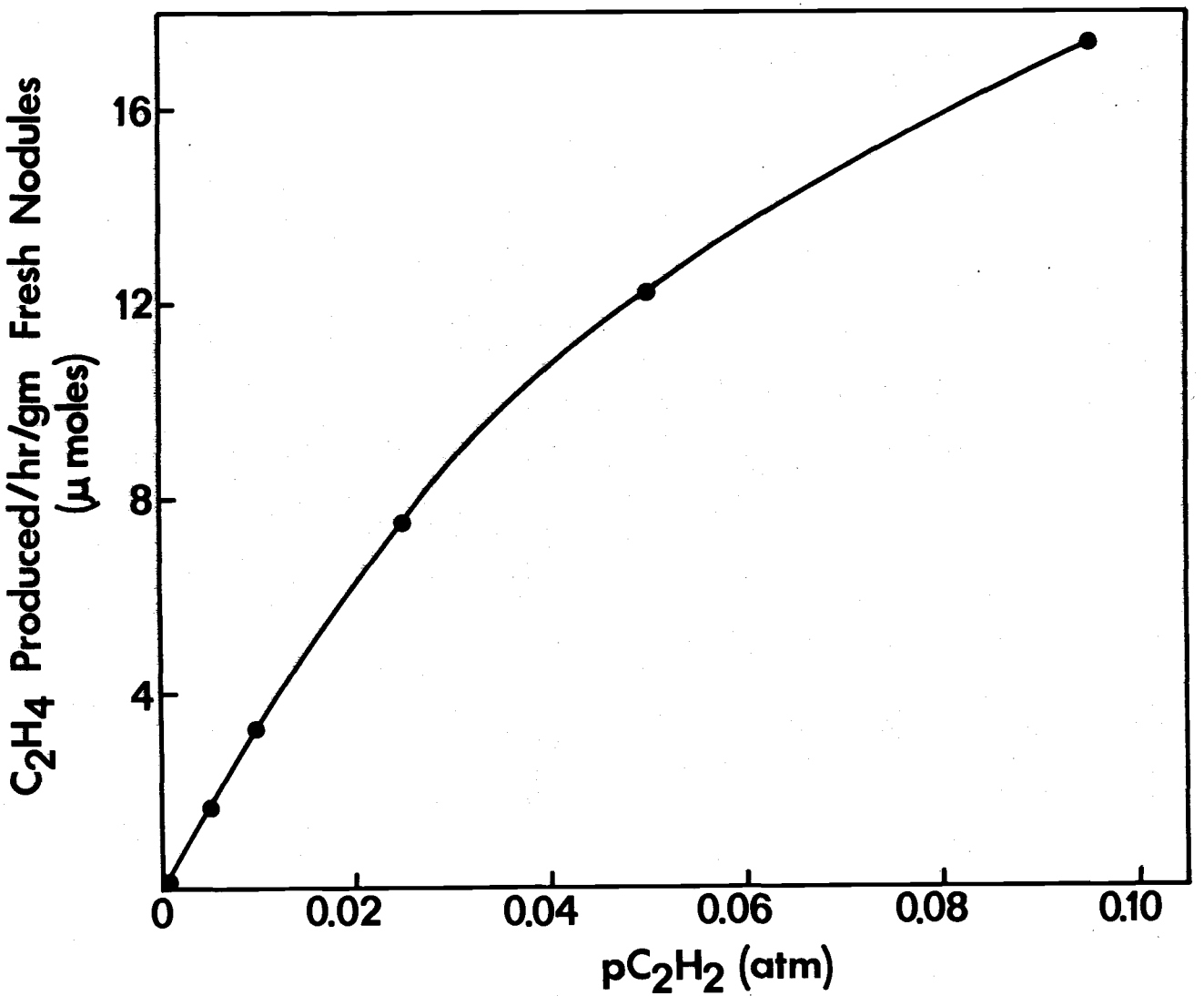

Figure 4. Saturation of nitrogenase with acetylene. Immediately following the assay, all of the nodules from a single culture were removed and fresh weights were determined.

Correlation Between Ethylene Production and the Fresh Weight of Nodules

The results of an experiment comparing the acetylene reducing capacity of three, six and nine plants per culture (Figure 5) showed that the amount of ethylene produced was apparently directly proportional to the fresh weight of the nodules. When the rates of ethylene production were plotted against the fresh weight of the nodules, a linear relationship was observed (Figure 6). When nodules were obtained from plants of different ages, the relationship between acetylene reduction rates and nodule fresh weight exhibited greater variability (Figure 7). A regression line was fitted to the data showing the relation between nodule fresh weight and ethylene production of plants at different ages (Figure 7). The correlation coefficient calculated on the basis of nodule fresh weight versus rate of ethylene production was 0.79, and on the basis of nodule dry weight versus rate of ethylene production was 0.70. These values along with the obvious simplicity of obtaining nodule fresh weights justify the usual practice of reporting acetylene reducing activity on the basis of the fresh weight of nodules (Hardy et al., 1968).

These results agree well with previous reports by other investigators. Chen and Thornton (1940) recognized a direct relationship between nodule size and the rates of nitrogen fixation. According to these workers, large nodules possess a greater percentage of tissue

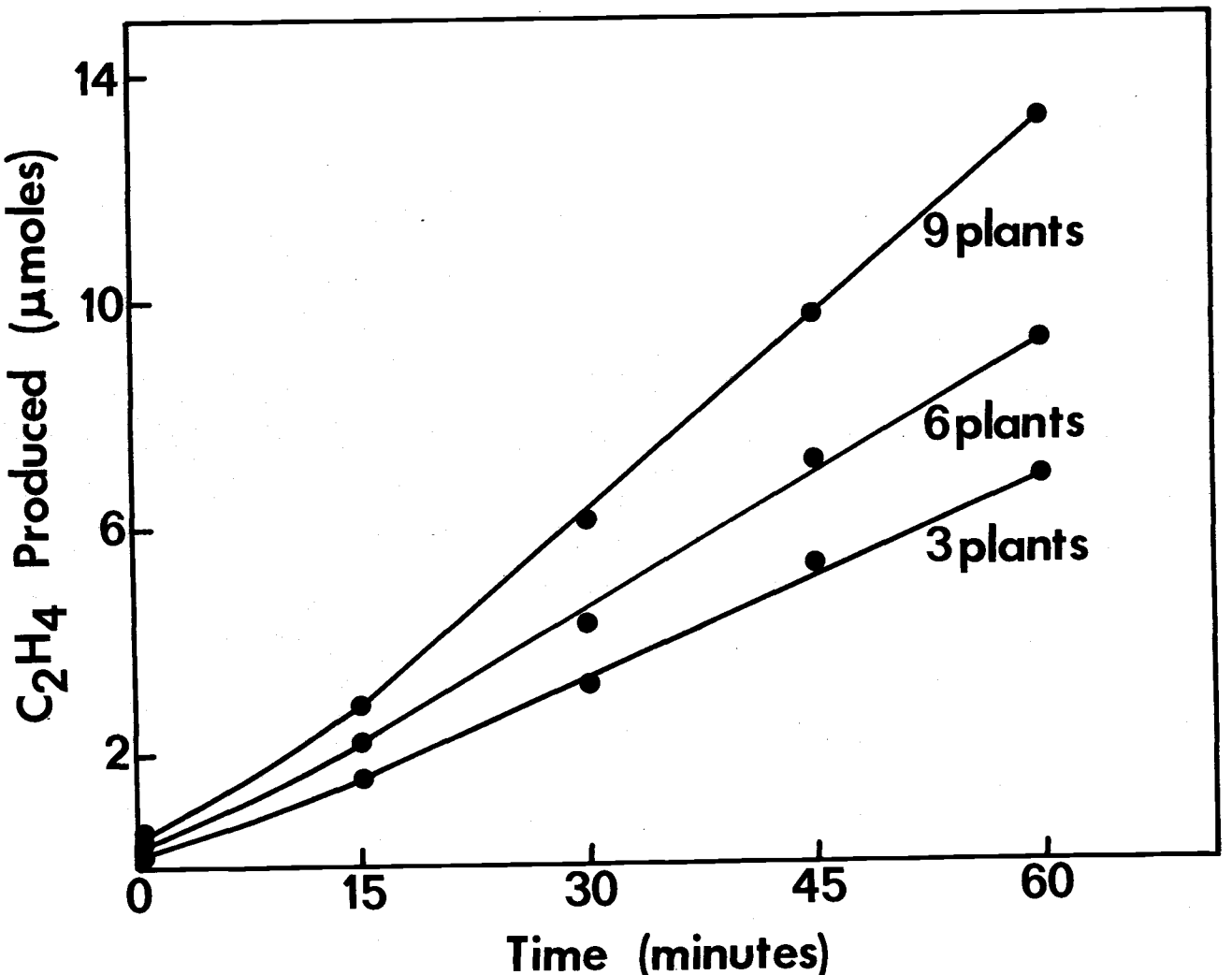

Figure 5. Relationship between rates of acetylene reduction and number of plants per culture. Assays were run on cultures which had been thinned to nine, six and three plants per pot.

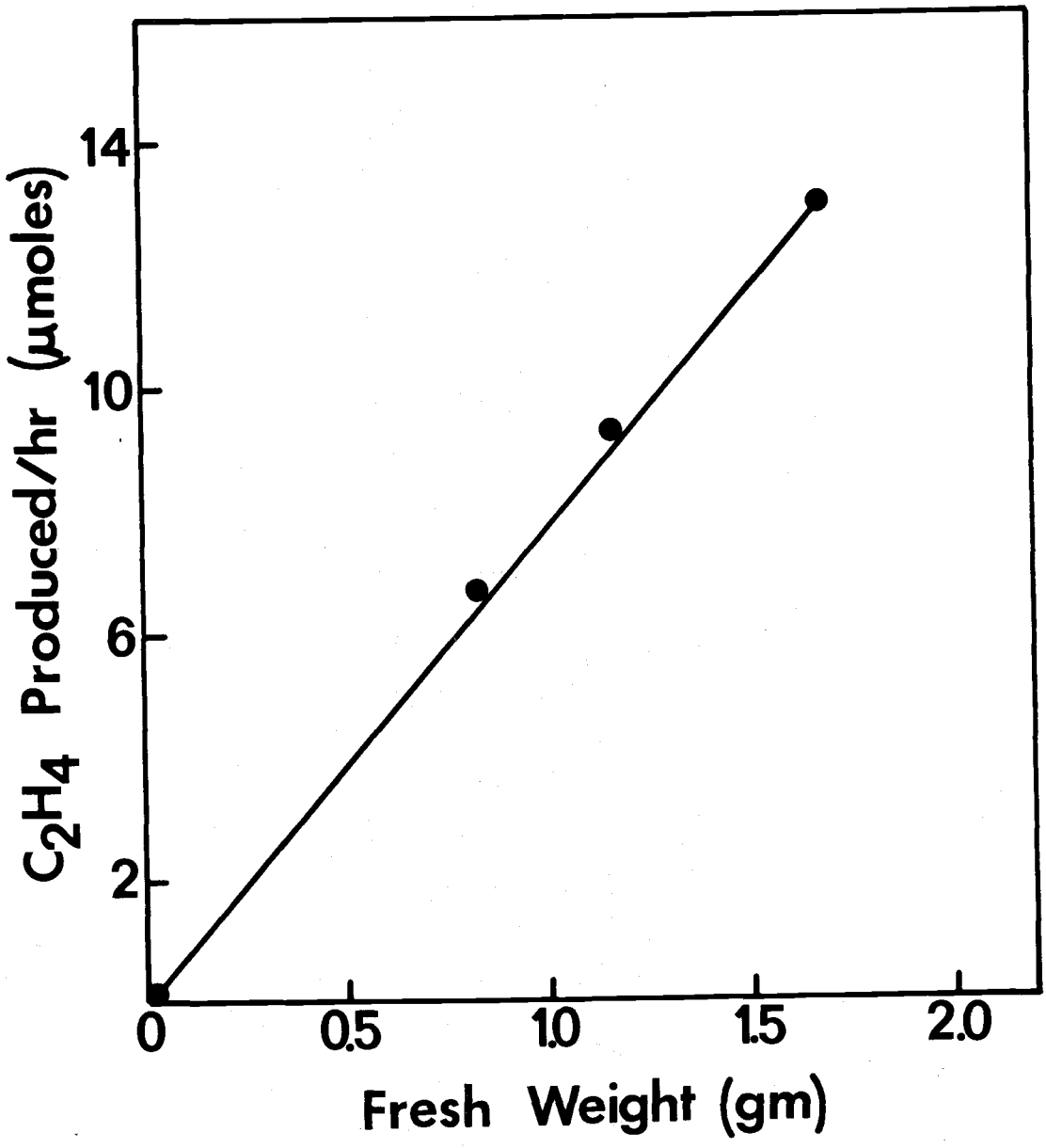

Figure 6. Correlation between rates of acetylene reduction and the fresh weight of nodules from plants of the same age. Immediately following the assay (Figure 5), all of the nodules from a single culture were removed and fresh weights were determined.

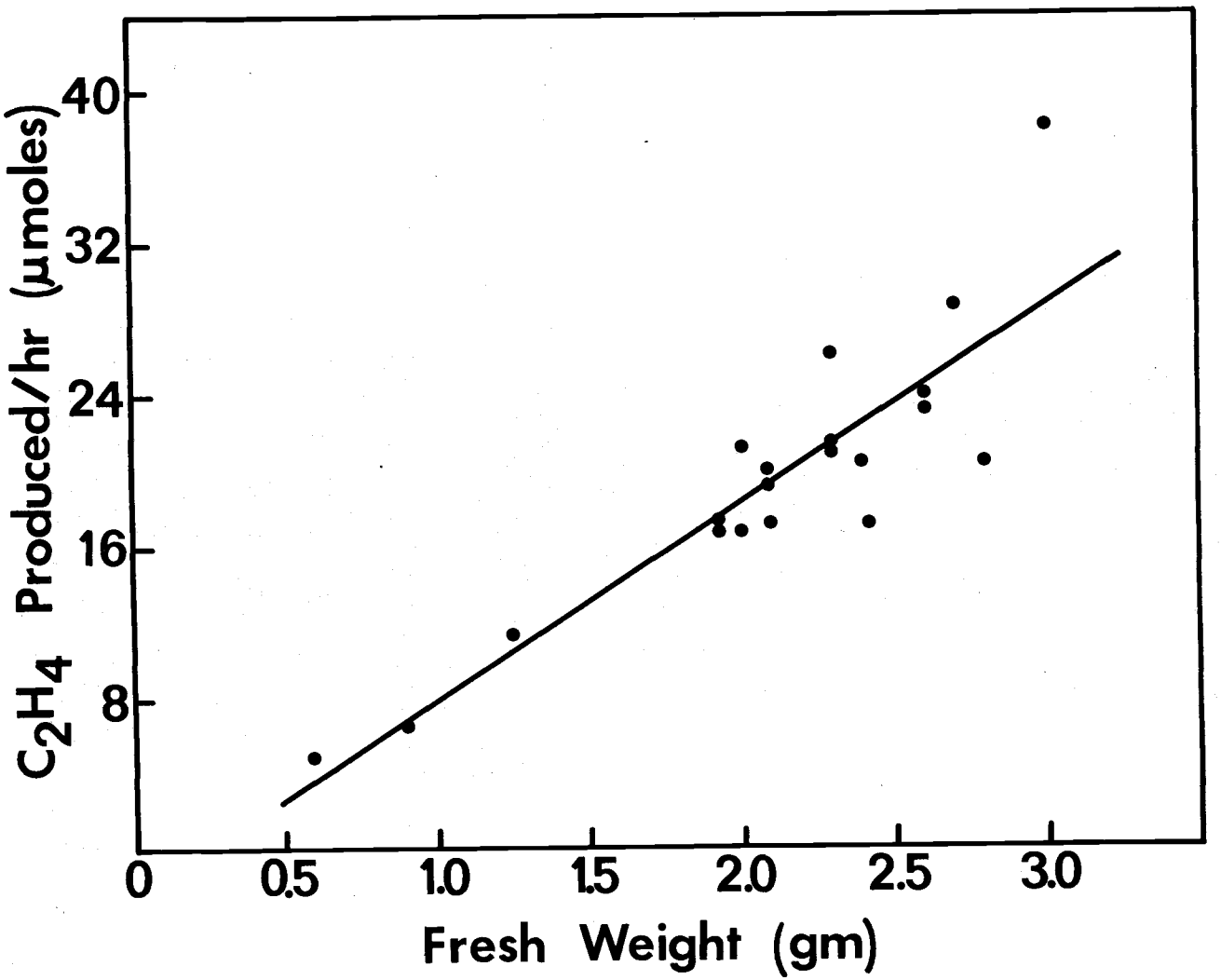

Figure 7. Correlation between rates of acetylene reduction and the fresh weight of nodules from plants of different ages. Immediately following the assay, all of the nodules from a single culture were removed and fresh weights were determined. A regression line was fitted to the data.

active in nitrogen fixation than smaller nodules. Aprison et al. (1954) arrived at the same conclusions. Nodules 5-6 mm in diameter fixed approximately twice as much nitrogen as nodules less than four mm. Hardy et al. (1968) showed that sample variability due to differences in nodule weights was decreased significantly when acetylene reducing activity was expressed as nodule efficiency.

Acetylene Reduction by Detached Nodules, Nodulated Roots and Intact Plants

A comparison was made between the acetylene reducing activity of detached nodules, nodulated roots and intact plants (Table 1). No difference was observed between the rates of acetylene reduction by nodulated roots removed from the culture medium and intact plants in situ whereas there was approximately a 40% decrease in the activity of the detached nodules from a comparable culture. These results generally corroborate the previous reports by Hardy et al. (1968), Bergersen (1970) and Magué (1971). Injury to the nodules, exposure of the enzyme in the nodules to oxygen and removal of the nodules from their source of photosynthate may all be involved in this decreased activity.

Table 1. A comparison of acetylene reduction by detached nodules, nodulated roots and intact plants.

The standard assay procedure (Materials and Methods) was followed using the following sample materials: 1) Nodules, all the nodules from the plants in a single culture were removed and assayed; 2) Nodulated roots, nodulated roots from the plants in a single culture were excised at the cotyledons and assayed; 3) Intact plants, the intact plants in a single culture were assayed in situ.

Sample	Ethylene Produced (μmoles per hour per gram fresh nodules)	
	Experiment 1[1]	Experiment 2[2]
Detached nodules	8.46 ± 0.76	7.0 ± 0.68
Nodulated roots	12.73 ± 1.36	12.82 ± 0.78
Intact plants (in situ)	12.83 ± 4.40	13.50 ± 1.77

[1] Each value (followed by the standard deviation) represents the mean of three replicate cultures.

[2] Each value (followed by the standard deviation) represents the mean of four replicate cultures.

Investigation of a Possible Effect of Diurnal Variation on Acetylene Reducing Activity

Field studies by several investigators including Hardy et al. (1968), Stewart et al. (1967) and Mague (1971) revealed that nitrogen fixation and acetylene reduction by detached nodules, excised nodulated root systems and intact nodulated plants exhibited marked variation related to day length, light intensity, rain and air temperature. Hardy et al. (1968) found no significant diurnal variation when plants grown under controlled conditions were assayed. Results similar to those of Hardy et al. (1968) were obtained in this study for

plants maintained under a 16-hour photoperiod at 24°C and a dark period at 18°C (Figure 8). Although there was a slight decrease in ethylene production at 22:00 hours, two hours following the onset of the dark period, there was no significant variation over the 24 hour period. Thus, experiments run at different times during the 16-hour photoperiod apparently were not significantly influenced by sampling at different times during the photoperiod. Mague (1971) observed considerable changes in acetylene reduction 1-2 hours after abrupt changes in light intensity during a brief summer squall. Bergersen (1970) reported that ethylene production by detached nodules was influenced significantly by preharvest illumination.

Effect of pO_2 on Acetylene Reduction

Bergersen (1970) emphasized the need to simulate *in situ* conditions as nearly as possible when correlating acetylene reducing activity with nitrogen fixation. The assay procedure utilized in the present investigation represents a practical, close approximation of compliance with this important recommendation. Among the environmental factors which must be considered is the pO_2. Although several investigators have observed the O_2 requirement for nitrogen fixation and acetylene reduction by excised soybean nodules (Burris *et al.*, 1955; Bergersen, 1962, 1970; Koch and Evans, 1966; Mague, 1971), there is disagreement on the optimum pO_2 for maximum activity.

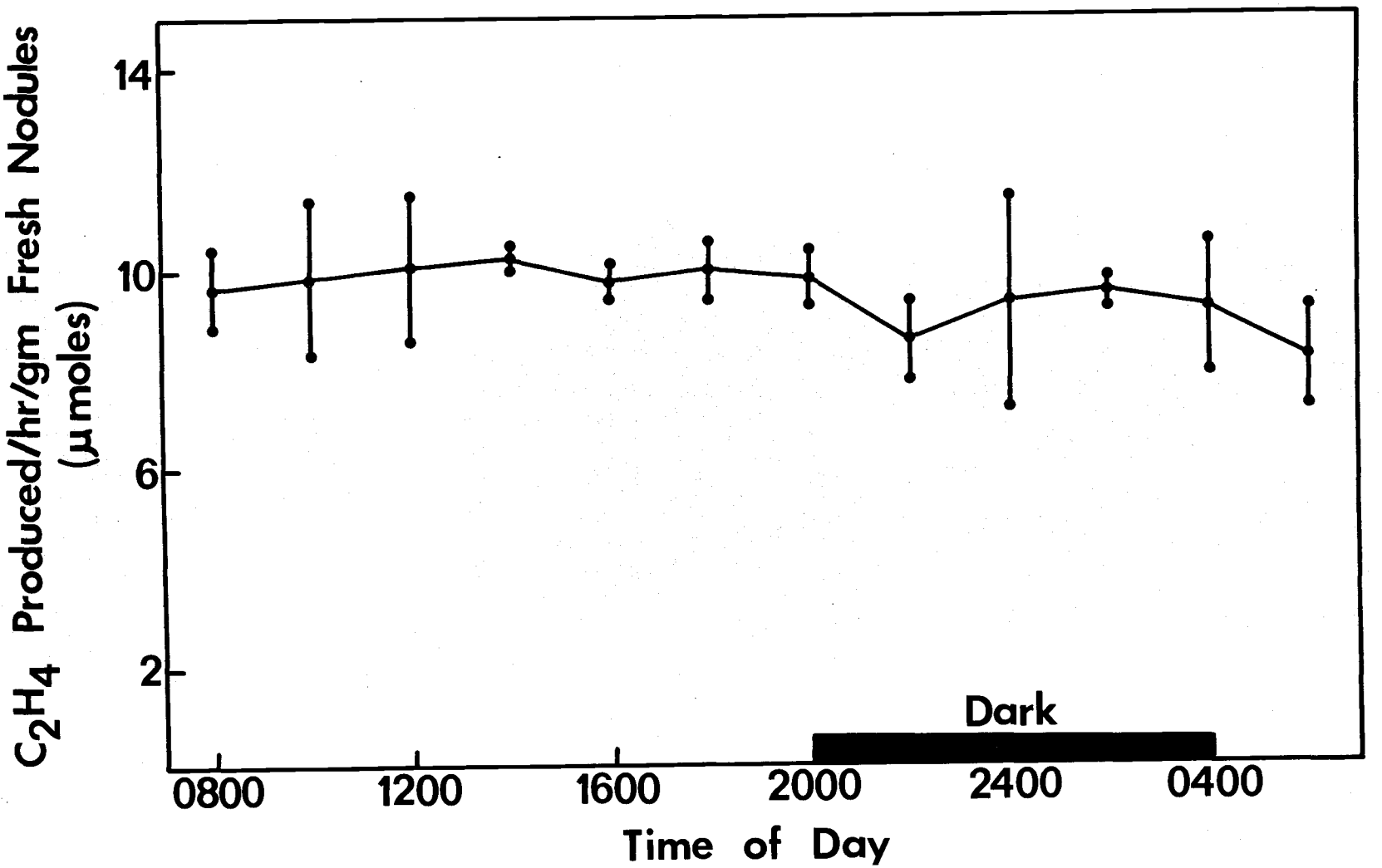

Figure 8. Investigation of a possible effect of diurnal variation on acetylene reducing activity. Immediately following each assay, all of the nodules from a single culture were removed and fresh weights were determined. Vertical lines represent the standard error of the mean.

Sprent (1969) reported that the maintenance of a large ratio of gas volume to nodule volume was essential to an adequate pO_2 for fixation and reduction. Optimum pO_2 concentrations do not necessarily represent conditions *in situ* as pointed out by Drozd and Postgate (1960) and therefore care must be taken when extrapolating from results obtained in the laboratory to natural conditions. The use of undisturbed intact plants and a large incubation chamber aid in simulating *in situ* conditions.

Applicability of the Assay to Soil-Grown Plants

To examine the feasibility of measuring the acetylene reducing activity of intact plants growing in a medium other than Perlite, soybean plants germinated and growing in Perlite (at approximately 10 days of age) were transplanted into soil and maintained at a moisture content approximating field capacity. In a time course experiment (Figure 9), ethylene production was linear throughout the 90-minute assay period. Since gas diffusion is approximately 10,000 times faster through air than through water (Meidner and Mansfield, 1968), it was speculated that the soil moisture content might be a significant factor in affecting the rate of acetylene reduction of intact cultures. Table 2 presents the average rates of ethylene production by six pots of plants maintained at three different water levels. These data indicate that moisture content has an effect on rates of acetylene reduction,

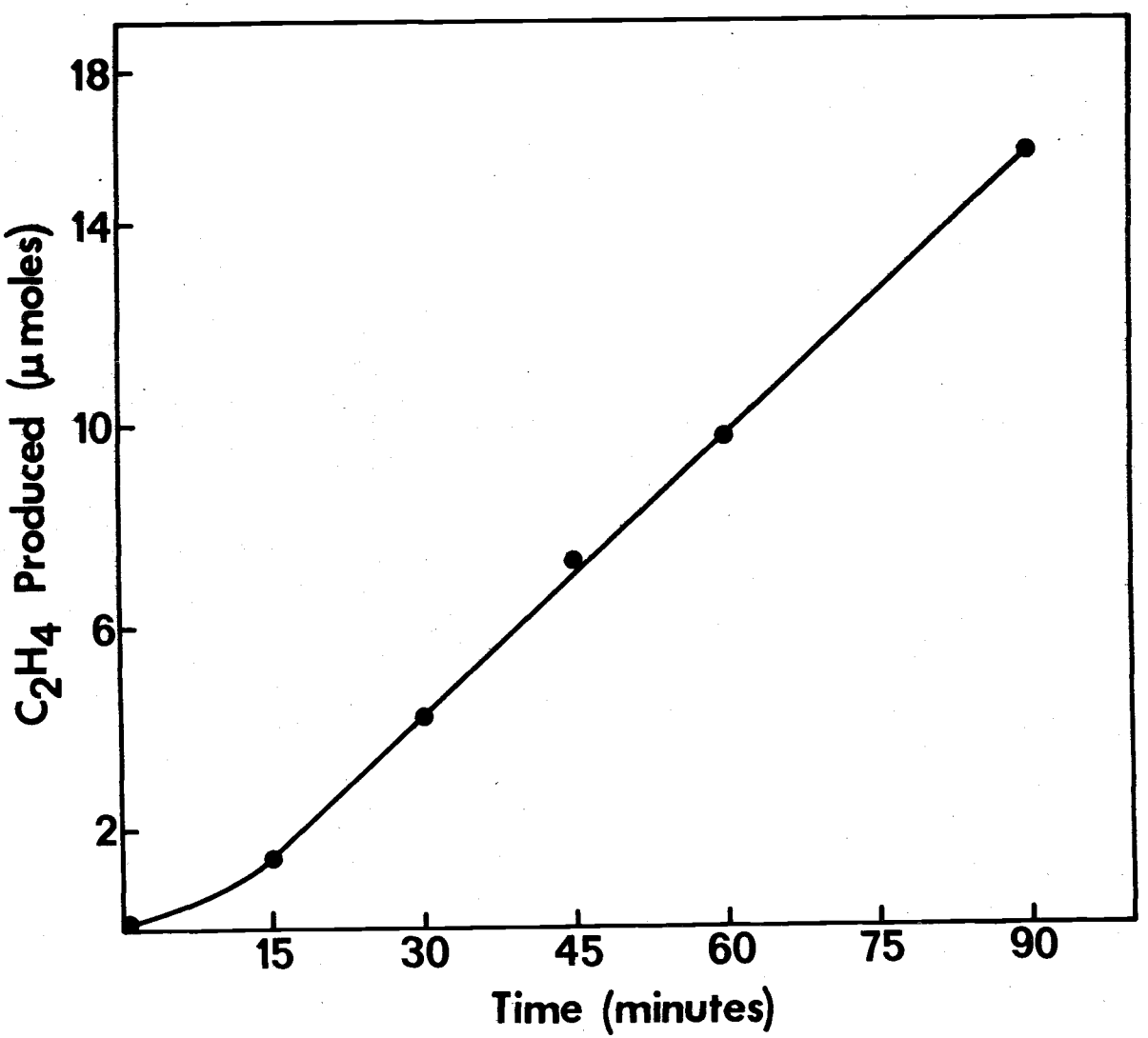

Figure 9. Time course of acetylene reduction by plants growing in soil.

but whether the effect is upon the reduction process or upon gas diffusion cannot be ascertained from this experiment.

Table 2. A comparison of acetylene reduction by intact plants grown in soil at three moisture levels.

The standard assay procedure (Materials and Methods) was followed.

Soil water suction (atm)	Ethylene produced (μmoles per hour)
0.15	2.75[1] ± 1.01
0.35	3.30[1] ± 0.56
2.00	7.46[1] ± 2.82
12.50 (wilted)	3.40[2] ± 0.84

[1] Value (followed by the standard deviation) represents the mean of six replicate cultures.

[2] Value (followed by the standard deviation) represents the mean of duplicate cultures.

In a subsequent experiment (Table 3), acetylene reduction rates by plants removed from their soil culture were compared with rates by plants in situ. The mean rate for plants removed from the soil was four times that of plants assayed in situ. It thus appears that the soil and its moisture content present barriers to gaseous diffusion. When the latter experiment was repeated using plants grown at three soil moisture levels, the effect of moisture on both the processes of reduction and diffusion was revealed (Table 4). The fact that plants grown at 0.60 atm (a water level below field capacity) produced 8.32

Table 3. A comparison of the rates of acetylene reduction by soil-grown plants *in situ* with nodulated plants removed from their soil culture ... soil maintained at field capacity.

The standard assay procedure (Materials and Methods) was followed. Intact nodulated plants were assayed either *in situ* or following removal from the soil. In the latter case excess soil was shaken from the nodules.

Material assayed	Ethylene produced (μmoles per hour per gram fresh nodules)[1]
Plants in soil	3.05 ± 0.64
Plants removed from soil	12.40 ± 1.13

[1] Each value (followed by the standard deviation) represents the mean of duplicate cultures.

Table 4. A comparison of the rates of acetylene reduction by soil-grown plants *in situ* with nodulated plants removed from their soil culture ... soil moisture content varied.

The standard assay procedure (Materials and Methods) was followed. Intact nodulated plants were assayed either *in situ* or following removal from the soil. In the latter case excess soil was shaken from the nodules.

Material assayed	Ethylene produced (μmoles per hour per gram fresh nodules)[1]		
	0.60 atm	0.15 atm	0.08 atm
Plants in soil	5.42 ± 1.99	2.67 ± 0.56	2.10 ± 0.39
Plants removed from soil	8.32 ± 1.74	13.05 ± 1.24	11.35 ± 2.02

[1] Each value (followed by the standard deviation) represents the mean of four replicate cultures.

μmoles C_2H_4 whereas plants grown under more moist conditions (0.15 and 0.08 atm) produced 13.05 and 11.35 μmoles C_2H_4, respectively, indicates that the water content of the soil has an effect on acetylene reducing activity of the nodules. On the other hand, the lower values obtained when plants grown under more moist conditions were assayed in situ reveals that there is also a measurable effect of the moisture content of the soil on gas diffusion.

In general, it has been observed by others that dehydration of nodule tissue results in lowered acetylene reducing activity. Sprent (1971a) found that reduction of the water content of nodules to 80% of normal fresh weight decreases the reduction of acetylene to ethylene and that irreversible changes occur when the fresh weight of the tissue drops below 80%. On the other hand, if excess moisture is present oxygen deficiency may occur resulting in lowered activity (Sprent, 1969).

CONCLUSIONS

The detection and measurement of nitrogenase activity have been significantly expedited by the introduction of the acetylene reduction assay. Extensive application of the technique to assaying nitrogenase activity in cell-free extracts, free living microorganisms detached nodules, nodulated root systems and intact plants has confirmed the biological validity and practical utility of the assay. This investigation has extended the use of the technique to the measurement of nitrogenase activity of intact plants in situ under conditions approximating natural environmental conditions.

In this investigation, a 21 liter polyethylene container large enough to accommodate intact plants rooted in Perlite or soil was employed as an incubation chamber. Moisture levels of approximately field capacity were maintained by irrigating the cultures prior to the experiments with a constant volume of nutrient solution or tap water at the same time every day. pC_2H_2 of 0.025 atmospheres was determined to be optimum for the experimental apparatus employed. Except for the time course experiments, all assays were run for 30 minutes. Under these conditions, constant rates of acetylene reduction to ethylene can be observed for whole plants in situ for periods up to two hours. Ethylene production measured under these conditions is proportional to the fresh weight of the nodules on plants in

equivalent physiological states. Variations in acetylene reduction due to diurnal variation in physical factors are precluded by preconditioning the plants to be assayed in a controlled physical environment.

Three very important recommendations should be borne in mind when applying the acetylene reduction assay, as described in this paper, to the measurement of nitrogen fixing activity.

First, environmental conditions should be simulated as nearly as possible, as indicated by the generally optimum conditions described in the preceding paragraph.

Second, extreme care should be taken in the procurement of representative samples. In comparison to the use of small containers, the large containers used in this investigation prevented altering the physiological state of the nodules and more nearly duplicated in situ conditions. In addition, the use of intact plants in situ eliminated any bias in sample selection as well as any damage to the nodule tissue as a result of removal from its normal physiological state. Although the polyethylene containers employed in this study are inexpensive, readily available and convenient for field studies, a serious disadvantage of their use should be noted. Their flexibility prevents the addition of gas volumes large enough to reach enzyme saturation and requires that extreme caution by taken to prevent gas leakage.

Third, the results of this investigation indicate that the acetylene reduction assay can be used to measure the nitrogenase activity of intact plants growing in soil as well as in a porous medium such as Perlite. However, if the assay procedure is to be used with plants rooted in soil, the moisture levels must be rigidly standardized to circumvent variations in apparent ethylene production due to variable rates of diffusion of gases through soils at different moisture tensions.

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