1975-04-01

An assay chamber for quantitative analysis of nitrogen fixation using intact plants

Thomas Adrie Leslie
Brigham Young University - Provo

AN ASSAY CHAMBER FOR QUANTITATIVE ANALYSIS OF NITROGEN FIXATION USING INTACT PLANTS

A Thesis

Presented to the

Department of Botany and Range Science

Brigham Young University

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

by

Thomas A. Leslie, Jr.

April 1975

ACKNOWLEDGEMENTS

I wish to express gratitude to Dr. Benjamin W. Wood, Department of Botany and Range Science, Brigham Young University, for suggesting the topic of this research and for his support, guidance, and encouragement which led to its completion.

Sincere thanks is also given to Que Adams and Tyler Rogers of the Instrument Shop for their assistance in solving the several technical difficulties which occurred during the initial construction and operation of the assay chamber developed as a result of this thesis.

Acknowledgement is also given to Dr. Kimball T. Harper, Chairman, Department of Botany and Range Science, for reviewing this manuscript and for his invaluable advice during the course of this research.

TABLE OF CONTENTS

LIST OF ILLUSTRATIONS

LIST OF TABLES

PURPOSE OF STUDY

The purpose of this investigation is to test the hypothesis that quantitative analysis of nitrogen fixation can best be accomplished by assaying "intact plants." Two goals of this study are: (1) to develop the laboratory apparatus necessary for assaying intact, greenhouse grown plants, and (2) to establish assay procedures for utilization of the above apparatus. Biological nitrogen fixation like photosynthesis and respiration is of primary importance for life. Since nitrogen is a component of many organic compounds essential for life, an understanding of the amounts of nitrogen fixed by symbiotic nitrogen fixers is desirable. Data obtained with the apparatus developed as a result of this study should allow more accurate measurements than has been possible in the past of the contributions of nitrogen fixers to the nitrogen economy of natural and agricultural systems.

INTRODUCTION

A persistent worldwide shortage of dietary protein has historically imposed the severest restrictions on human nutrition (Hardy et al., 1971). It was estimated in 1968 that some two billion people were not being properly fed. "Undernourishment and starvation stem not only from food shortage but also from supply of the wrong type of food--food that is rich in carbohydrates and deficient in proteins" (Stewart, 1967). A New Republic article (1965) estimated that five million children in India die each year of malnutrition. Protein deficient diets are mainly responsible for these deaths (Ehrlich, 1968). The worldwide protein shortage, in existence for many decades (Paddock, 1958), is basically a shortage of fixed nitrogen (Hardy and Knight, 1968). The need to produce more protein thus translates as a need for more nitrogen, the main sources of which are chemical or symbiotic and non-symbiotic biological nitrogen fixation (Hardy et al., 1971).

It is estimated (Donald, 1960) that 100 million tons of nitrogen are fixed annually on this planet, ninety percent of which is of biological origin. Except in highly industrialized nations where artificial nitrogen fertilizers are widely used, the rate at which symbiotic plants and free-living microbes return nitrogen to the soil determines the soil's productivity. Manufactured fertilizers trap N_2 and so do chemical fixation processes (e.g. oxides of nitrogen are generated by lightning, ultraviolet radiation, operation of internal combustion engines, volcanic activity, from the burning of organic matter, especially

coal, and from forest fires; the rain washes these into the soil as nitrates (Salisbury and Ross, 1969; Postgate, 1971); but taken together they accounted for only about 6 percent of the total nitrogen required for world food production in 1959 (Dhar, 1959). Biological nitrogen fixation then is the rate-determining step in food production on most parts of the planet (Postgate, 1971). However, the contributions of biologically fixed nitrogen to world agriculture is difficult to assess (Hardy et al., 1971). This difficulty can be attributed to the absence of effective methods for quantitatively measuring nitrogen fixation in situ (Hardy et al., 1968). Within the past few years, however, research in biological nitrogen fixation has begun to provide the technology essential for an expanded agronomic exploitation of this important process. Since the work of Dilworth (1966) and Schollhorn and Burris (1967) showed that acetylene was reduced to ethylene by nitrogen fixing systems, a number of researchers have developed the phenomenon as a sensitive assay for nitrogen fixing activity (Koch and Evans, 1966; Stewart et al., 1967; Hardy et al., 1968; Moustafa, 1967 and 1969; Roughley and Dart, 1969; Sprent, 1969; and Hardy et al., 1971). The acetylene assay provides a useful means for indirect, immediate evaluation of biological nitrogen fixation. The method is extremely rapid, has a very low control or background correction for ethylene, is specific for ethylene and is not limited by expensive or unique equipment or materials such as isotopic N_2^{13} and N_2^{15} assays (Stewart et al., 1967; Hardy et al., 1968). Its validity as an index of nitrogen fixation has been verified by direct comparison with N_2^{15} (Stewart, 1967) and micro-kjeldahl analysis (Hardy et al., 1968 and 1971). Most studies utilizing the acetylene assay have been done in relation to agriculturally significant crops,

and understandably enough. These studies have involved specific biochemical investigations of nitrogen fixation and have as a goal the enhancement and extension of symbiotic nitrogen fixation to major crops. This would significantly improve crop yields, thus relieving some of the need for artificial nitrogen fertilizers, while at the same time minimizing the problems of high distribution cost and indirect coupling of nitrogen-to-plant that are associated with these fertilizers (Hardy et al., 1971). Presently, however, world agriculture is already highly efficient. So that the prospect of massively increasing world food supplies on existing agricultural lands are dim (Ehrlich, 1968). As a need for more food develops, marginal lands, i.e., desert soils and other wildlife ecosystems, will be brought into production. Only recently have desert soils been studied for utilization to meet the world's growing food needs (Rychert and Skujins, 1973). As an extension of this thesis it might be possible to identify native nitrogen fixers adapted to these marginal lands. Then those plants could be established on similar sites where they are deficient, thereby increasing land fertility and production. As a result, more protein would be available to meet increased food needs throughout the world.

Most of the investigators utilizing the acetylene reduction technique, have worked with excised nodules (Koch and Evans, 1966; and Hardy et al., 1968 and 1971), attached nodules of decapitated plants (Moustafa, 1969; and Wood, 1971) or cell-free extracts (Carnahan et al., 1960). In some cases, plants grown in greenhouses have been used to evaluate the nitrogen fixing capacity of whole plants by measuring the amount of total nitrogen produced per unit of biomass (Russel and Evans, 1966). Using these methods they have attempted to measure the nitrogen

fixing capabilities of plants and their rate of fixation. Then by extrapolation from laboratory data, they have "estimated" the contribution of selected species to the nitrogen economy of the specific ecosystem in which they are found. The acetylene assay is designed to assess in situ activities, and therefore, the reaction environment should duplicate in situ conditions rather than provide optimum laboratory conditions for nitrogen fixation (Hardy et al., 1971). However, most sample preparations currently in use, begin by decapitating plants at the soil line. Next, the root system is removed from the soil and the nodules are either completely excised from them (Koch and Evans, 1966) or are left attached to small pieces of root (Moustafa, 1969). Samples are then transferred to a 50 ml syringe assay chamber. Although this assay method has been valuable in improving estimates of the quantity of N_2 fixed under natural conditions, these in situ studies are really more applicable to investigations of free-living soil organisms and lichens which can be placed, relatively undisturbed, into these small assay chambers. Experimental difficulties have been encountered by those seeking to demonstrate fixation of nitrogen by excised nodules (Aprison and Burris, 1952). Removal of nodules from the roots and their subsequent storage, for even short periods, results in a lowered (Schwinghamer, 1970) or complete loss (Moustafa, 1969) of their nitrogen fixing ability. Moustafa (1969) used excised root samples with attached nodules. He found that by leaving small pieces of root attached to the nodules, there was no significant loss in the rate of acetylene reduction up to 30 minutes of storage. He also notes that whole plants could be stored in plastic bags up to 4 hours without significant loss in the ability of their nodules to reduce acetylene. The decrease in rate or

complete loss of ability of excised nodules to fix nitrogen with time suggests that an intermediate (i.e. possibly a photosynthetic substrate) apparently supplied to the nodules by the host plant is being used up or that the enzyme (nitrogenase) reducing nitrogen and acetylene, is being inactivated (Aprison and Burris, 1952). In addition to the above problems, removal of the organism to be assayed from its native soil and its subsequent transportation to the laboratory certainly places it under abnormal stresses, e.g., light, temperature, moisture, oxygen, etc.

Previous studies utilizing the acetylene reduction assay have not investigated "intact" plants in relation to nitrogen fixation. It is felt, that in order to obtain a better understanding of nitrogen fixation, it is important to investigate nitrogen fixers for their total effect on the nitrogen economy of their respective ecosystems. This cannot be satisfactorily accomplished by determining whether isolated nodules can fix nitrogen. Therefore, there is a need for a tool which can analyze nitrogen fixation by "intact" plants. The successful development of such an instrument would allow accurate determinations of the nitrogen contributions of symbiotic and non-symbiotic nitrogen fixers to the nitrogen economy, thus eliminating the need to make interpretations by extrapolation of laboratory data, as has been done to present. Several researchers agree that there is a need to be able to go into the field with a suitable instrument and measure the rate of nitrogen fixation by "intact" plants in their natural environment (Allison, 1965).

LITERATURE REVIEW OF BIOLOGICAL NITROGEN FIXATION

History

Without realizing the source of the benefits, early Greek and Roman farmers rotated leguminous and nonleguminous crops because of the observation that nonleguminous crops grew more vigorously following legumes. Crop rotation was recognized as good husbandry, but an explanation of the benefit was not given until the late 1830's (Burris, 1974). In 1838, J. Boussingault, the French scientist-farmer, utilizing the ocular assay to analyze field experiments reported that legumes could utilize atmospheric nitrogen (Burris, 1965). This conclusion was contested for over forty years by such eminent scientists as the German chemist J. Von Liebig (Burris, 1974). Further experimentation was performed in an effort to decide who was right, Boussingault or Liebig. Experiments of a French scientific commission favored Boussingault's conclusions, while the experiments of Lawes et al., in 1861, strongly agreed with Liebig's conclusions that leguminous nitrogen fixation did not occur. The experiments of Laws et al. were performed so carefully that they appeared definitive in concluding Liebig was correct. However, there were a few skeptics such as W. O. Atwater who continued to report evidence of nitrogen fixation by legumes (Burris, 1974). In 1885, W. O. Atwater published results in the American Chemical Journal of the first experiments on biological nitrogen fixation undertaken in the United States (Wilson, 1963). Atwater's experimental data showed that he had obtained proof of nitrogen fixation by pea plants. However,

he hedged observing ". . . that the plants (peas) assimilate free nitrogen is contrary to general belief and to the results of the best investigations of the subject" (Atwater, 1885). The definitive experiments of Hellreigel and Wilfarth in 1886 were required to establish biological nitrogen fixation by leguminous plants (Burris, 1965). Hellriegel and Wilfarth were interested in the nitrogen nutrition of plants. Their experiments included control plants to which no nitrogen was added. They demonstrated that some legumes flourished in the nitrogen-free medium of the control plants. In every instance those which did well in the absence of nitrogen had nodulated root systems.

Leguminous root nodules had been observed for centuries, but no one had guessed that they were responsible for nitrogen fixation. They also suggested that microorganisms were responsible for nodule formation. The experiments of Atwater (1885 and 1886) and Hellriegel and Wilfarth (1886) utilized a total nitrogen assay for determining that plants grown in nutritive solutions exposed to the air but protected from rain and dew, contained more nitrogen than was supplied them in nutritive solution and seed (Atwater, 1885; and Hellriegel and Wilfarth, 1888).

In 1888, Beijerinck isolated the bacterium Rhizobium from the root nodules of a leguminous plant (Waksman, 1927). This timely isolation of a species of Rhizobium confirmed the observation of Hellriegel and others that some kind of microorganisms located within the root nodules of legumes were in all probability active agents in assimilation of molecular nitrogen. Concurrently, Winogradsky demonstrated that certain of the free-living anaerobes, e.g., Clostridium pasteurianum, could fix gaseous nitrogen (Burris, 1965).

In 1894, Schneider reported dichotomously branched, coraloid-shaped root nodules, on Cycas revoluta. The function of the nodules of Cycas revoluta and other Gymnospermae still remains to be determined (McKee, 1962; and Stewart, 1967). One possibility is that of nitrogen fixation. Bond (1959) reported that detached nodules of Ceratozamia and Encephalartos spp. exposed to the N_2^{15} assay (Burris et al., 1942 and 1943) gave evidence of fixation, while roots alone without nodules showed no fixation. Silver et al. (1963) have also demonstrated nitrogen fixation by an isolated endophyte (a species of Klebsiella) in leaf nodules of Psycotria bacteriophylla.

Early studies on both nodulated legumes and nonleguminous Angiospermae and Gymnospermae were often done by the same investigators (Allen and Allen, 1965). However, because of the greater economic importance of leguminous species, the study of nonleguminous nitrogen fixers was practically discontinued until Hawker and Fraymouth's work in 1951. Since then, nodulation has been reported and verified, utilizing various assays, for about 100 species in 8 families of the Angiospermae, with nodulation also being documented for several members of the Gymnospermae (Allen and Allen, 1965). Recently, there have been reports of several new species, both leguminous and nonleguminous, which may be involved in nitrogen fixation (Allen and Allen, 1965; Farnsworth and Hammond, 1968; and Holiday, 1971).

Assays

Quantitative methods of assaying biological nitrogen fixation have steadily improved since the initial use of the micro-kjeldahl technique (Hardy and Knight, 1968). Historically, organisms were screened for the detection of nitrogen fixing activity by the ocular assay. This

test for the ability to fix nitrogen was simple--will organisms grow on a nitrogen-free medium (Wilson, 1951)? If they did, then they must be obtaining the nitrogen necessary for growth from the atmosphere by biological nitrogen fixation (Atwater, 1885). Though not a quantitative method the ocular assay was valuable because it stimulated the investigation of many plant species for their ability to fix nitrogen. There were several serious limitations effecting results obtained by this method. Atwater (1886) reported that nitrogen was liberated from its compounds contained in germinating seeds and the mature plant by fermentation, volatilization, etc. This tends to reduce the amount of nitrogen found at the end of the experiment and thus, the quantity apparently obtained from the air by nitrogen fixation. A second source of error involved with this method, is that in order to obtain accurate results, the assay must be made in the absence of any source of combined nitrogen. Early researchers found it very difficult to obtain truly nitrogen-free culture media. Many organisms grew quite well on minute traces of organic nitrogen present in the so-called nitrogen-free medias in use then. Similarly, sufficient quantities of nitrogenous compounds were available in the atmosphere, which permitted limited growth by several organisms (e.g., bacteria, algae) during the lengthy experimental periods which often lasted weeks or months (Mortenson, 1961). It became evident that a technique was needed which would eliminate these particular errors and allow a quantitative analysis of the nitrogen fixing ability of a given organism. "If ocular assay suggests that a particular organism can fix N_2, N_2-fixing ability can be established by showing that its final content of nitrogen is greater than that supplied from the medium and surrounding air is fixed nitrogen during the period of testing"

(Burris and Wilson, 1957). The micro-kjeldahl analysis (Kjeldahl, 1883) and its various modifications (Bradstreet, 1940; Vickery, 1946; Hiller, et al., 1948; and Ballentine, 1957) was the first quantitative method used in an attempt to verify results of ocular assays. Originally, the method was designed for the brewing industry as an aid in following protein changes in grain during germination and fermentation (Kjeldahl, 1883). Its use soon became widespread and was utilized by Atwater (1885 and 1886), Hellriegel and Wilfarth (1886), and others to assay nitrogen fixation. Basically, it is a wet oxidation employing concentrated sulfuric acid as the digestion medium, and resulting in the formation of ammonium sulfate, which is subsequently distilled with an excess of alkali (Bradstreet, 1940). The ammonia thus formed is used to determine total nitrogen in biological materials, utilizing various titrametric procedures (Ballentine, 1957). Although the Kjeldahl assays are simple in principle, errors inherent in the techniques (Ballentine, 1957; and Wilson, 1951) and difficulties in obtaining uniform samples make these methods applicable only to organisms which fix substantial quantities of atmospheric nitrogen (Wilson, 1951; and Burris and Wilson, 1957). Using the Kjeldahl assay, it is impossible to accurately detect increases in the total nitrogen of a sample, of less than one percent (Wilson and Burris, 1957). Inaccuracies of the Kjeldahl method have frequently suggested fixation of nitrogen by germinating seeds, Rhizobium sp. independent of its host, non-leguminous plants and other agents whose ability to fix nitrogen is questionable (Burris and Miller, 1941). The limitations of the various micro-Kjeldahl procedures are well known to chemists and biologists as evidenced by the numerous articles in the literature dealing with the "best" conditions, e.g., time, heating and catalysts,

for obtaining accurate results with the various Kjeldahl techniques (e.g., Bradstreet, 1940; Hiller et al., 1948; Hallet, 1948; and Ballentine, 1957). As with the ocular assay, the Kjeldahl analyses are affected by the quantity of nitrogen originally present in the media and in the surrounding atmosphere (Wilson, 1951). Test organisms, e.g. whole cells or intact plants, must be grown under conditions in which ammonia and nitrogenous compounds are excluded from the culture media and air. The limitations of the several micro-Kjeldahl procedures do not mean that the results reported are necessarily incorrect, only that precise conclusions cannot be made from them. For example explanations other than nitrogen fixation can often be used to account for significant increases in total nitrogen (Wilson, 1951). Because of the problems inherent with Kjeldahl techniques, several workers developed direct methods for demonstration of nitrogen fixation by gasometric analysis (e.g., Virtanen, 1939, Hurwitz and Wilson, 1940, and Allison et al., 1942). These methods measure uptake of molecular nitrogen directly. However, these procedures were time consuming, laborious and not nearly as reliable or precise as N_2^{15} isotopic assays.

Prior to the development of the N_2^{15} isotopic assay (Burris and Miller, 1941; Burris 1941, and Burris et al., 1942 and 1943), quantitative analysis of biological nitrogen fixation was based upon analysis for total nitrogen by Kjeldahl assays or decreases in gas concentrations by gasometric methods, neither of which were reliable enough to detect small increases in the nitrogen content of materials initially high in nitrogen (Smyth and Wilson, 1935; and Wilson et al., 1939). The N_2^{15} isotopic procedure is not subject to the limitations of either of the previously described assays (Wilson, 1951). N_2^{15} analysis is relatively

independent of the nitrogen present in the media or original test materials and is not subject to the procedural errors encountered with the Kjeldahl assay. This is because fixation is determined by the percent increase of the N_2^{15} tracer and not the total nitrogen content of the sample. The test organism is grown on any suitable medium in an atmosphere containing excess N_2^{15}. After incubation, the nitrogen in the sample is first converted into ammonium sulfate by a convenient Kjeldahl procedure and then into molecular nitrogen by a basic hypobromite. Samples of molecular nitrogen are then analyzed by a suitable isotope ratio type of mass spectrometer. By extensive replication and appropriate statistical analyses an increase in total nitrogen of one percent may be detected with the Kjeldahl method (Burris and Wilson, 1957), whereas with a mass spectrometer as little as 0.003 atom percent N^{15} excess can be detected and an incorporation of 0.015 atom percent excess above the normal abundance of N^{15} in the test organism or greater is acceptable for establishing nitrogen fixation (Burris and Wilson, 1957; and Hardy and Knight, 1968). This method is at least forty times as sensitive as micro-Kjeldahl procedures for establishing nitrogen fixation (Burris and Wilson, 1957). This assay has been used extensively in determining fixation in whole cells (Burris et al., 1943), root nodules (Aprison and Burris, 1952), and cell-free extracts of nitrogenase (Carnahan et al., 1960). Although, this assay has been used for analysis of samples exposed to N_2^{15} in the field to demonstrate nitrogen fixation <u>in situ</u>, it has three disadvantages: (1) it is time consuming, (2) few laboratories are equipped with a mass spectrometer, and (3) N_2^{15} is expensive (Wilson, 1951; and Hardy and Knight, 1968). These factors have limited its widespread application in field studies (Hardy et al.,

1968).

A second isotopic assay utilizing N_2^{13} as a tracer was developed in 1961 by Nicholas et al., 1961. N_2^{13} produced by a cyclotron is freed of ammonia and nitrogen oxides before incubation with whole cells or cell-free extracts (Nicholas et al., 1961; Campbell 1966; and Campbell et al., 1967). This method is at least one-hundred times as sensitive as the N_2^{15} assay. However, as with the N_2^{15} method expensive equipment, limited facilities for isotopic production, coupled with the short half-life (10.05 min.) of isotopic N^{13} makes its use in determining biological nitrogen fixation very impractical (Hardy and Knight, 1968; and Burris, 1974).

A modivied Conway microdiffusion technique (Mortenson, 1961) was used to titrametrically measure the ammonia produced by nitrogenase in cell-free extracts during nitrogen fixing experiments. Experimental evidence shows that this method is comparable to the N_2^{15} assay. This method has advantages over the procedures previously described, since results can be obtained as soon as one hour after completion of incubation. However, it is relatively insensitive, i.e., about one-twentieth as sensitive as the N_2^{15} technique (Hardy and Knight, 1968). A similar manometric procedure was again used to assay cell-free extracts by Mortenson in 1964. Due to the opportunity for other types of errors and their insensitivity, neither has been widely used as assays of biological nitrogen fixation.

In 1967, the opportunity arose for a sensitive new assay for detection of biological nitrogen fixation, based on the versatility of the reductive catalyst nitrogenase (Hardy et al., 1967; and Hardy and Knight, 1968). It was determined that nitrogenase would catalyze the

reduction of a number of triply bonded compounds besides nitrogen, including N_2O, N_3, alkyenes, HCN, nitriles and isonitriles (Hardy and Burns, 1968). In addition, Dilworth (1966) and Schollhorn and Burris (1967) found that acetylene (C_2H_2) was reduced to ethylene (C_2H_4) by nitrogenase in a reaction analogous to the reduction of nitrogen to ammonia. The application of this reaction to a new sensitive assay for biological nitrogen fixation was first proposed by Hardy and Knight (1967). Ethylene formed from acetylene by nitrogenase is detected with a hydrogen flame after separation by gas chromatography.

It has been found that acetylene reduction by nitrogenase has the same requirement for reductant and ATP as does the reduction of nitrogen to ammonia. The activities in terms of reduction of acetylene or nitrogen were parallel during purification of nitrogenase from free-living bacteria and from legume nodule bacteroids (Hardy et al., 1968). When energy and reductants are not limiting the ratio of C_2H_2:N_2 reduced is 3:1 (Klucas, 1968; and Hardy et al., 1971). Values close to this theoretical value have been obtained by several researchers with cell-free extracts of nitrogen fixers but there is little information available from intact plants (Bergersen, 1970). The acetylene reduction assay has proven to be extremely sensitive (about 2×10^{-8} g/L or 10^{-12} mole per assay). It is at least as rapid as the Conway microdiffusion procedure, has a very low control or background correction for ethylene (Bergersen, 1970), and its application is not limited by expensive or unique equipment as are the N_2^{15} or N_2^{13} assays (Hardy and Knight, 1968). The only other method with equivalent or greater sensitivity is N_2^{13} incorporation; however, because of the disadvantages previously discussed, the use of N_2^{13} is restricted to limited investigations. Since

the original proposal of Hardy and Knight (1967) a number of workers (e.g., Koch et al., 1967 (a,b), Monstafa et al., 1969; Bergersen, 1970; and Hardy et al., 1971) have successfully employed acetylene reduction, coupled with ethylene detection by hydrogen flame gas chromotography, as an assay for biological nitrogen fixation (Hardy et al., 1968). Experiments such as these have established the validity of the acetylene reduction method for nitrogenase activity (Bergersen, 1970).

With this method it now becomes possible and practical to conduct comprehensive surveys of nitrogen fixation, to make detailed comparisons among different nitrogen fixing symbionts, determine the effectiveness of various rhizobial strains, and to rapidly evaluate the effects of agricultural practices and environmental factors on nitrogen fixation. The knowledge obtained through extensive application of this assay should provide the basis for research leading to the maximum agricultural exploitation of biological nitrogen fixation and a better understanding of the nitrogen cycle (Hardy et al., 1968). The measurement of the input of nitrogen into ecosystems, prior to development of the acetylene reduction assay was a major problem (Bergersen, 1970; and Hardy et al., 1971). Utilization of the acetylene reduction method will allow accurate measurement for this purpose and will also be invaluable for the measurement of nitrogen fixation <u>in situ</u> (Hardy et al., 1968 and 1970). In a 1974 review article Burris reports that the acetylene reduction technique ". . . has been of real use in improving estimates of the quantity of N_2 fixed under natural conditions."

Since the development of the acetylene reduction technique as a simple assay for measuring N_2 fixation other researchers have tried to develop assay methods using N^{15}/N^{14} natural abundance ratios (Delwiche

and Steyn, 1970). However, they concluded that ". . . it is unlikely that any quantitative conclusions can be drawn from isotope ratio data concerning the extent to which nitrogen has been cycled from the atmosphere to the soil and back."

Processes of the in situ acetylene reduction to ethylene assay are described in detail by Hardy et al. (1968 and 1970). All steps in their assay procedures are designed to minimize sample alteration and to establish in the assay chamber a micro-ecosystem that is identical to the macro-ecosystem from which the sample is obtained (Hardy et al., 1968). Currently, nodulated leguminous species are sampled by the complete removal of the root system with attached nodules (Hardy et al., 1968 and 1971; Monstafa 1969; and Bergersen, 1970) or by a soil bore directly over the tap root (Hardy et al., 1968; and Monstafa et al., 1969). Detached nodules were used in earlier investigations (Aprison and Burris, 1952; Silver, 1967; and Stewart et al., 1967) but they were found less active in time course fixation studies.

Even under the best conditions, using nodulated roots, time course studies show continuing acetylene reduction only up to about sixty minutes, then decreasing abruptly (Hardy et al., 1968; Monstafa, 1969; and Wood, 1971). This decrease may be attributable to several factors, e.g., the decrease may be due to oxygen depletion in the assay chamber during incubation (Hardy et al., 1968) or to depletion of photosynthetic products (Bergersen, 1970) (i.e., carbohydrates or other unknown compounds; Salisbury and Ross, 1969) which are then unavailable to nodulated roots after decapitation. Nitrogenase is a fairly unstable system (Bergersen, 1970) and failure to adequately match in situ conditions in the acetylene reduction assay with those of the nitrogen-fixing

system in nature may have an effect on the results obtained. It is apparent then, that after decapitation of a plant and the subsequent removal of its root system from the soil, maintenance of *in situ* conditions during the assay period is impossible. Micro-ecosystems which are supposedly identical to the macro-ecosystems, from which samples are obtained for use in the assay methods devised by Hardy et al. (1967), are not measuring fixation under true *in situ* conditions. One then wonders how closely estimates of fixation obtained from excised nodules relates to fixation under field conditions. Therefore, there is a need for a tool to analyze biological nitrogen fixation by intact plants in their natural environment (Allison, 1955).

MATERIALS AND METHODS

Assay Chamber

Design and construction of the assay chamber began in September 1973 and was completed in December 1974. The main features of the assay chamber include a hemispherical plexiglass cuvette, a cylindrical steel base, a refrigeration and heat exchange system, an environmental control and visual readout system, and a sample injection system. Air temperatures within the cuvette can be controlled to either simulate ambient air conditions or be held constant at any desired temperature (see results section for detailed discussion of these features).

Growth of Legumes

Soybeans (Glycine max Merr. var. Merrit) (supplied by W. Atlee Burpee Co. Seed growers Riverside, California) were innoculated with a commercial strain of Rhizobium (supplied by The Nitragin Company, Inc., Milwaukee, Wisconsin 53209) and cultured in 8-inch clay pots containing a 1:1 mixture of sterilized, quartz sand, and perlite. The plants were maintained in a greenhouse at a day temperature of approximately 25°C and 17°C at night. During the winter months supplemental light was furnished with fluorescent lights to maintain a day-night regime of 14 and 10 hours respectively. Each pot was flushed daily with 500 mls of a half-molar strength nitrogen-free nutrient solution (Machlis and Torrey, 1956, p. 44). Iron was supplied as an iron complex of sodium ferric ethylenediamine di-o-hydroxyphenyl acetate, containing 5.945 percent Fe ("Sequestreme 138 Fe," Geigy Chemical Corp.). Excess nutrient solution

drained through a single hole in the bottom of each pot.

The potted soybeans were kept on a 4 x 8 foot sheet of plexiglass, elevated at one end, which allowed the excess effluent, from the drain holes of the pots, to flow to the lower end where it was guided to a catchpot by a short length of galvanized gutter. The solution in the catch pot was maintained at the original volume with distilled water and was recycled through each experimental pot for five days, then each pot was flushed with distilled water to remove any accumulated salts. A fresh nutrient solution was prepared every 30 days.

Assay of Acetylene and Ethylene

Nitrogen fixation was assayed in situ utilizing a modified acetylene-ethylene reduction assay technique. Intact, potted soybeans were placed in the assay chamber. Acetylene gas (1.2 liters) was introduced into the assay chamber at a rate of 40 mls/minute and the plants were allowed to incubate for at least forty hours. One milliliter samples were injected into a Carle Model 211, analytical gas chromatograph for detection of acetylene reduction. A 3/16 inch (O.D.) x 4 foot long Porapak N gas chromatography column was used to perform separation of acetylene from ethylene. Porapak N is composed of porous polymer beeds which provides sharp symmetrical peaks and rapid separation of C_2H_2 from C_2H_4. Retention times in minutes for acetylene and ethylene were 30 and 24 seconds respectively.

A standard calibration gas containing 100 ppm ethylene (supplied by Altech Associates, 202 Campus Drive, Arlington Heights, Ill.) was used to derive a standard curve in order to determine the concentration of ethylene produced.

Calibration Procedures

It is important that the assay chamber be gas tight since long incubation periods are necessary for assays of biological nitrogen fixation. In order to ensure that the system was leak-free, the empty assay chamber was filled with 1200 milliliters of acetylene gas and then sampled after 20 and 40 hours had elapsed. Two replications were made for the 20 hour period and one for the 40 hour period. Periodically one milliliter samples of acetylene gas from the chamber were injected into an analytical gas chromatograph in order to detect any change in concentration which would indicate gas loss. The area under the gas chromatographic peaks were accurately determined using a rolling disc planimeter (A. Ott Kempton Rolling Disc Planimeter, W. Germany). The mean and standard deviation were then calculated from these areas.

Experimental controls were run to ensure that ethylene production occurred only in the presence of nodulated legumes. The empty chamber, wet and dry samples of the legume culture medium, and the clay pots in which the soybeans were grown were each tested for acetylene reduction. Pots with the culture medium, but without plants, which were watered in the same manner as those pots with plants were also tested for their ability to fix or absorb acetylene.

Initially it was assumed that after an arbitrary period of 20 hours the experimental plants and chamber gases would have equilibrated sufficiently so that fixation could occur. However, after the first two plants were incubated for 20 hours maximum fixation was not observed and an actual decrease in fixation was noted for plant No. 1 (see Table 2). The decrease may have been due to a diffusion effect. A second set of control experimental data was then collected for 20 hour periods and

compared to that obtained after 40 hours, a diffusion effect was noted. Apparently more than 20 hours are required for the environment within the chamber to become stabilized. Therefore because of convenience, periods of approximately 40 hours were selected as the incubation period before assays were made.

Data from the gas chromatograph using the standard ethylene gas (100 ppm) were compared with data obtained using acetylene gas which was obtained from industrial sources. The areas under the curves produced by the strip chart recorder were determined using a rolling disc planimeter with an arm ratio set at 2.82. Since these areas were relative and used only for comparison, these data were multiplied by 1000 in order to work with whole numbers.

Experimental Procedures

When they were approximately 35 days old, greenhouse grown soybeans were placed in the assay chamber *in situ* and allowed to incubate for at least 40 hours. The atmosphere within the chamber was a mixture of room air and 1200 milliliters of acetylene gas. Analyses of 1 milliliter samples of the gas mixture within the assay chamber were analyzed periodically in an analytical gas chromatograph for detection of initial production and any increase in the concentration of ethylene gas which would indicate occurrence of biological nitrogen fixation.

At the end of the incubation period, experimental plants were removed from their pots and the root systems were washed in order to remove soil particles and any root nodules present were removed. Fresh and dry weights were obtained for the nodules to provide a basis for determining the amount of nitrogen fixed and to compare these *in situ* results with *in vitro* acetylene reduction studies. The entire plant plus

excised nodules was oven dried at 40°C for 24 hours. Dry weight of the entire plant and nodules was obtained so that fixation might be expressed per gram of biomass.

RESULTS AND DISCUSSION

The Assay Chamber

A diagrammatic representation of the assay chamber and its associated instrumentation system is shown in Figure 1. The main features of the assay chamber include a hemispherical plexiglass dome, a cylindrical steel base pan, a refrigeration and heat exchange system, an environmental control unit and visual temperature readout system, and a sample injection system (Figure 2).

The Cuvette

The cuvette (Figure 3) is a transparent hemispherical plexiglass dome (Plastic Fabricating & Supply, Inc., Murray, Utah - special order) 87 cm in diameter and 44 cm in height with a thickness of 7 mm. The dome is positioned on a cylindrical steel base pan and held in place by 50 mm "C" clamps. A rubber gasket 7 mm thick, permanently affixed to the 38 mm flanged lip of the upper edge of the base pan, ensures a gas tight seal between the dome and base.

The Base

The base (Figure 4) is a sheet of hot rolled steel 3.05 meters long, 192 mm wide and 3 mm thick. It was formed into a cylinder with a 38 mm perpendicular flange lip around the top of the cylinder. A circular piece of 7 mm plate was cut to form a bottom for the cylinder. Two handles were cut from 20 mm steel rods. All parts were arc welded ensuring gas tight seams. The finished base pan is a cylinder 91 cm in

diameter and 17 cm in height. The total air volume of the dome and base is approximately 270 ± 0.5 liter. Eight holes (five 14 mm and three 7 mm) were drilled in the side wall of the base permitting installation of three sampling ports, one acetylene gas entry port, two coolant hoses, two environmental control thermistors, one thermocouple, and two electrical power connections (Figure 5). All necessary entry and exit port were located in the sidewall of the steel base to allow complete detachment of the plaxiglass dome. This permitted easy introduction of experimental plants, soils etc., within the assay chamber and protects the cuvette from possible damage due to drilling.

Refrigeration and Heat Exchange System

A White-Rogers temperature control refrigeration unit (Figure 6) was used to maintain two gallons of ethylene glycol (anti-freeze) (Figure 7) at 10°C. A Teel inline pump (Figure 8) (Dayton Electric Mtg. Co. Chicago) is used to pump the chilled coolant into the dome through a 14 mm flexible plastic tube to the heat exchange unit.

The heat exchange unit consists of a 19 cm square aluminum radiator (the core of a car heater) mounted on an aluminum base with a 78 mm fan on one side (Figure 9). The fan blows the air within the closed assay chamber across the radiator coils, cooling it to a desired temperature. Throughout this experiment the air temperature inside the assay chamber was kept at a constant 22°C.

The refrigerated coolant is pumped to the heat exchange unit and thus any heat accumulating inside the assay chamber is removed as the warmed coolant is returned to the refrigeration unit.

Environmental Control Unit and Visual Readout

Two linear thermistors and one thermocouple (Figure 10) are located inside the assay chamber. One of the thermistors detects air temperature within the assay chamber giving a visual readout (a TRI-R Electric Thermometer, Figure 11). The thermocouple is responsible for regulating the refrigeration and heat exchange system so that a constant temperature is maintained. Figure 11 also shows the environmental control box (below TRI-R thermometer) to which the thermocouple is connected. This controller calls for coolant to be pumped to the heat exchanger when needed as indicated by the thermocouple. A second thermistor is available for placement in soil samples, under plant leaves, etc., as needed. It also registers a visual readout.

Injection System

There is one 40 mm long, 0.5 mm diameter efflux air duct, and one 50 mm long, 0.5 mm diameter return air duct positioned adjacent to each other in the side-wall of the cylindrical steel base. The two air ducts are connected to the 1 milliliter sample valve of the gas chromatograph by flexible plastic tubing (Figure 12). The samples of the chamber atmosphere are pumped out of the chamber to the gas chromatograph via a Hush II aquarium pump (Metaframe Corp., Maywood, N.J., Figure 13).

Sampling can also be done through a rubber diaphram located in the side-wall of the cylindrical steel base of the assay chamber using a syringe and needle (see Figure 5). Syringe samples can then be injected directly onto the gas chromatographic column for separation.

Fig. 1. Schematic diagram of the assay chamber used for acetylene reduction assay studies. Dashed lines with arrows represent flow of water-ethylene glycol mixture from the refrigeration system to the heat exchange unit. Solid lines with arrows indicate flow of sample gas to gas chromatograph. The "T's" are sensors allowing visual observation of soil and air temperature. T_1 is a thermocouple by thich the automatic environmental controller operates (it detects changes in air temperature and maintains a constant temperature within the assay chamber). The other two are thermistors.

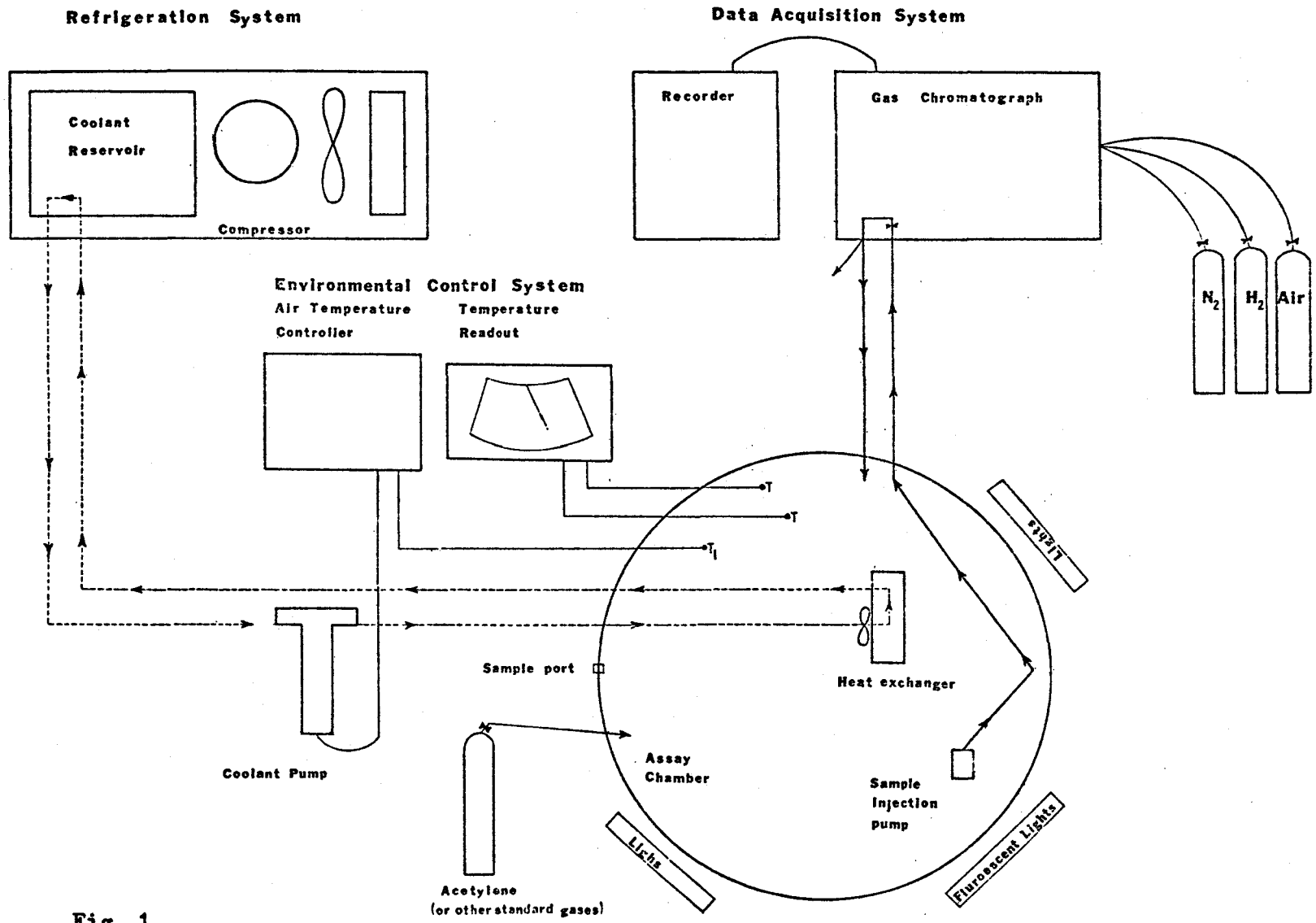
Refrigeration System
Data Acquisition System
Coolant
Reservoir
Compressor
Recorder
Gas Chromatograph
N_2
H_2
Air
Environmental Control System
Air Temperature
Controller
Temperature
Readout
T
T
T_1
Lights
Sample port
Heat exchanger
Coolant Pump
Assay
Chamber
Sample
Injection
pump
Lights
Fluorescent Lights
Acetylene
(or other standard gases)

Fig. 1.

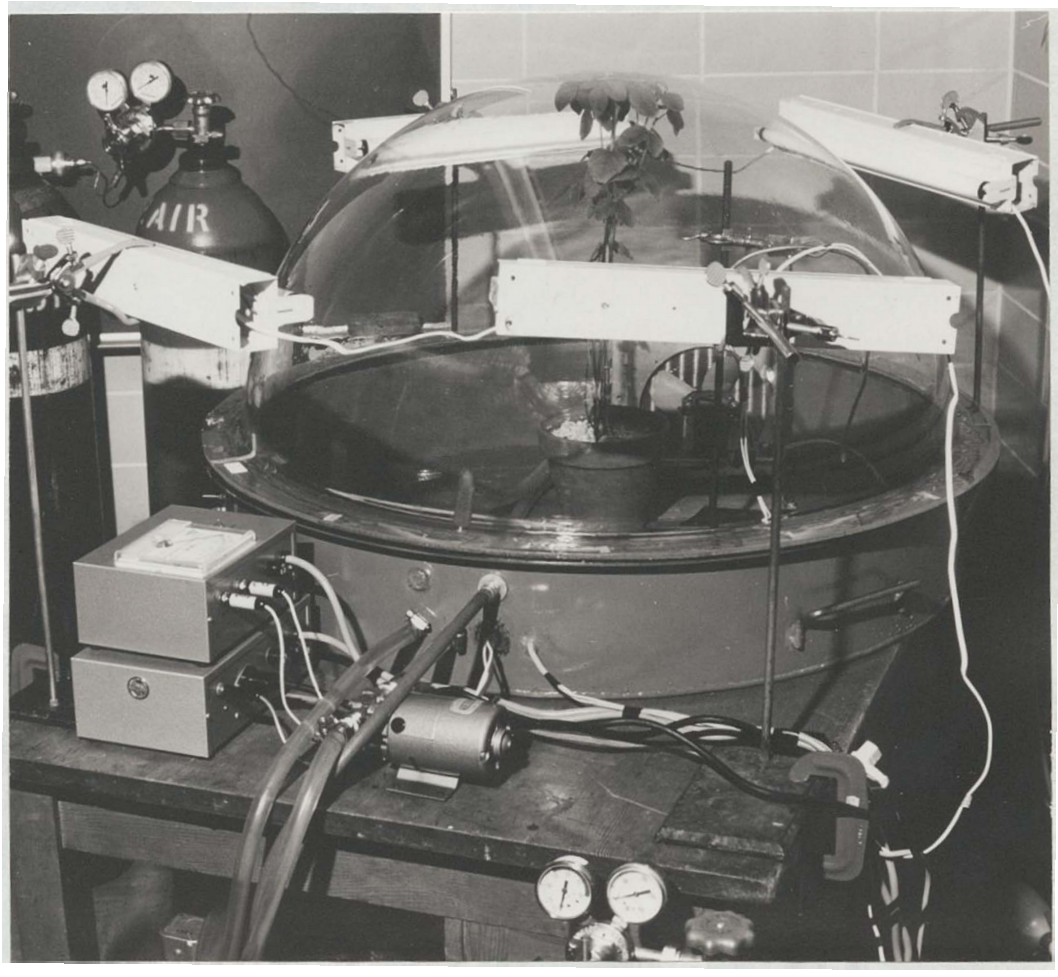

Fig. 2. Assembled assay chamber with associated instrumentation system.

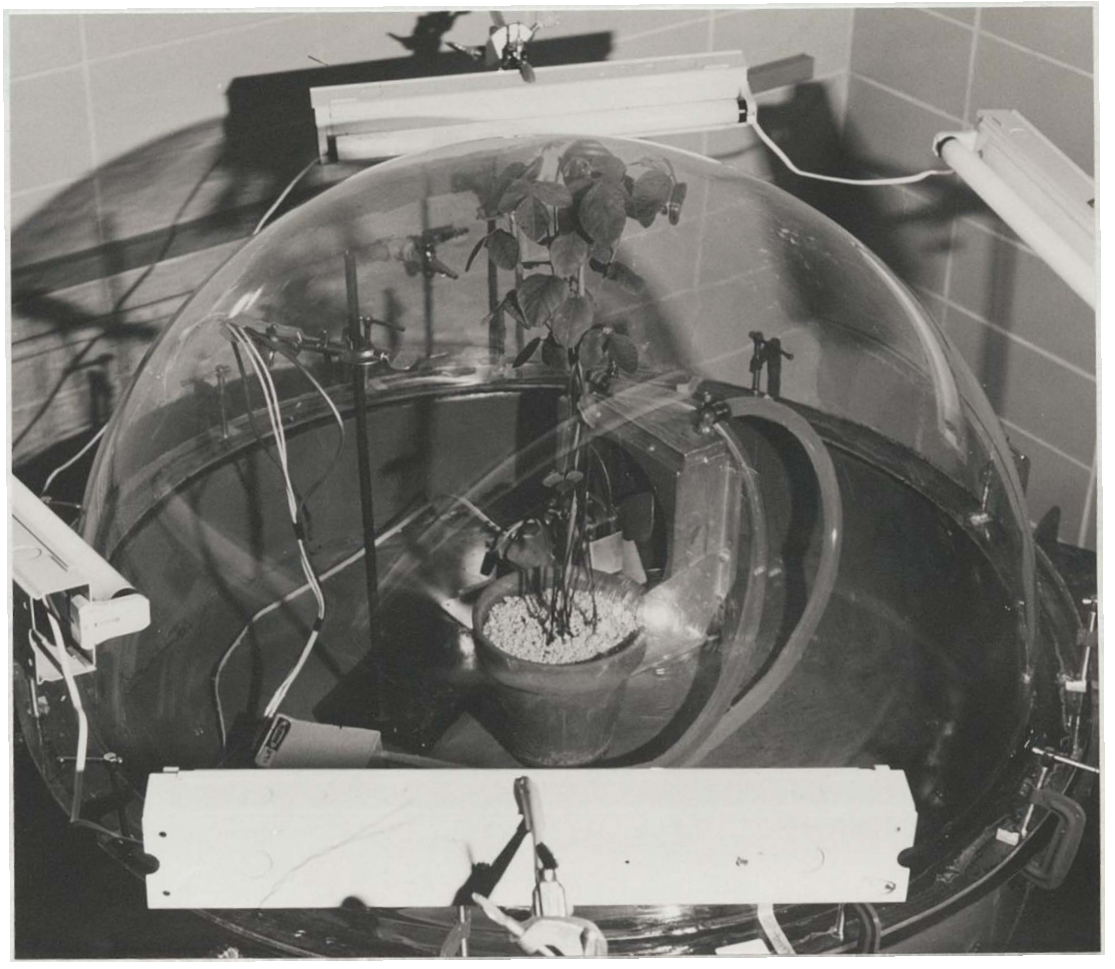

Fig. 3. Hemispherical plexiglass dome.

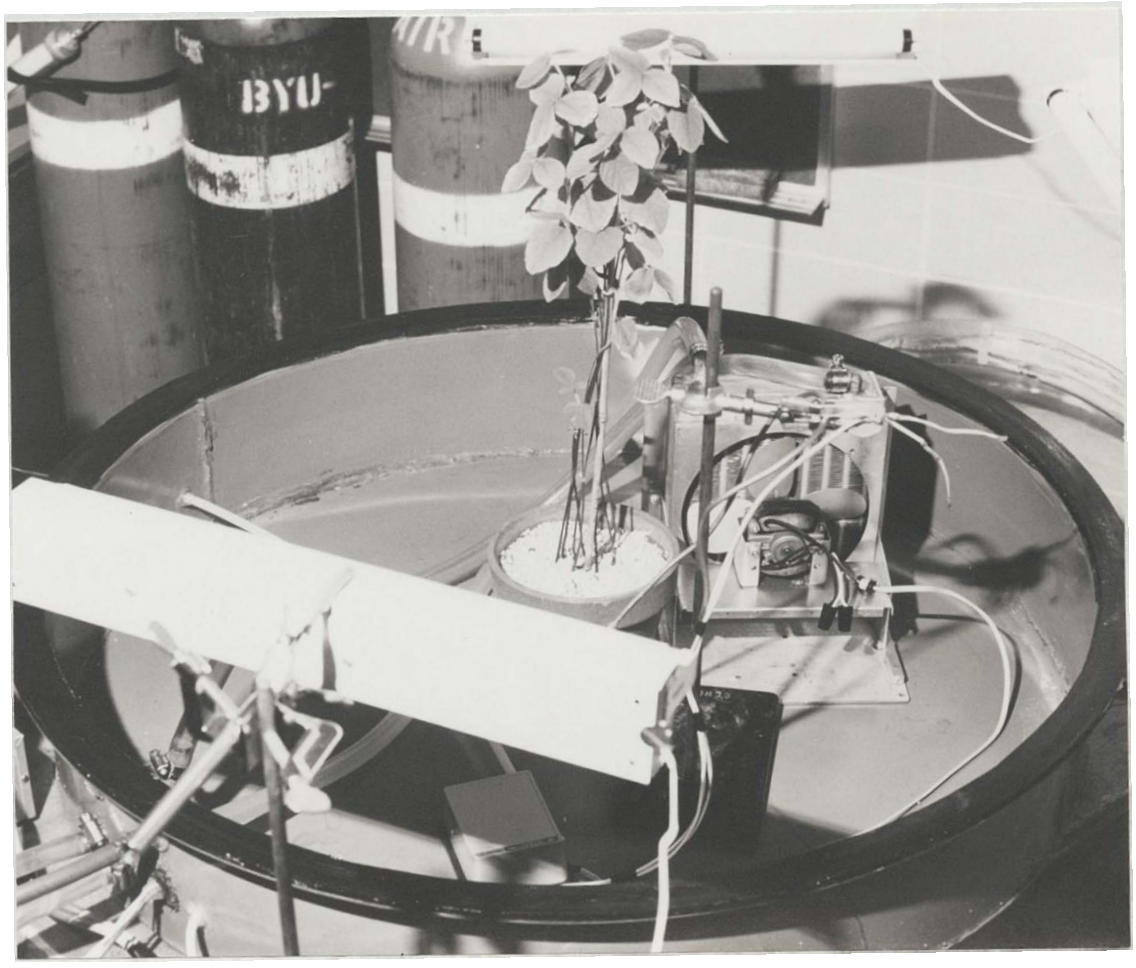

Fig. 4. Cylindrical steel base of assay chamber.

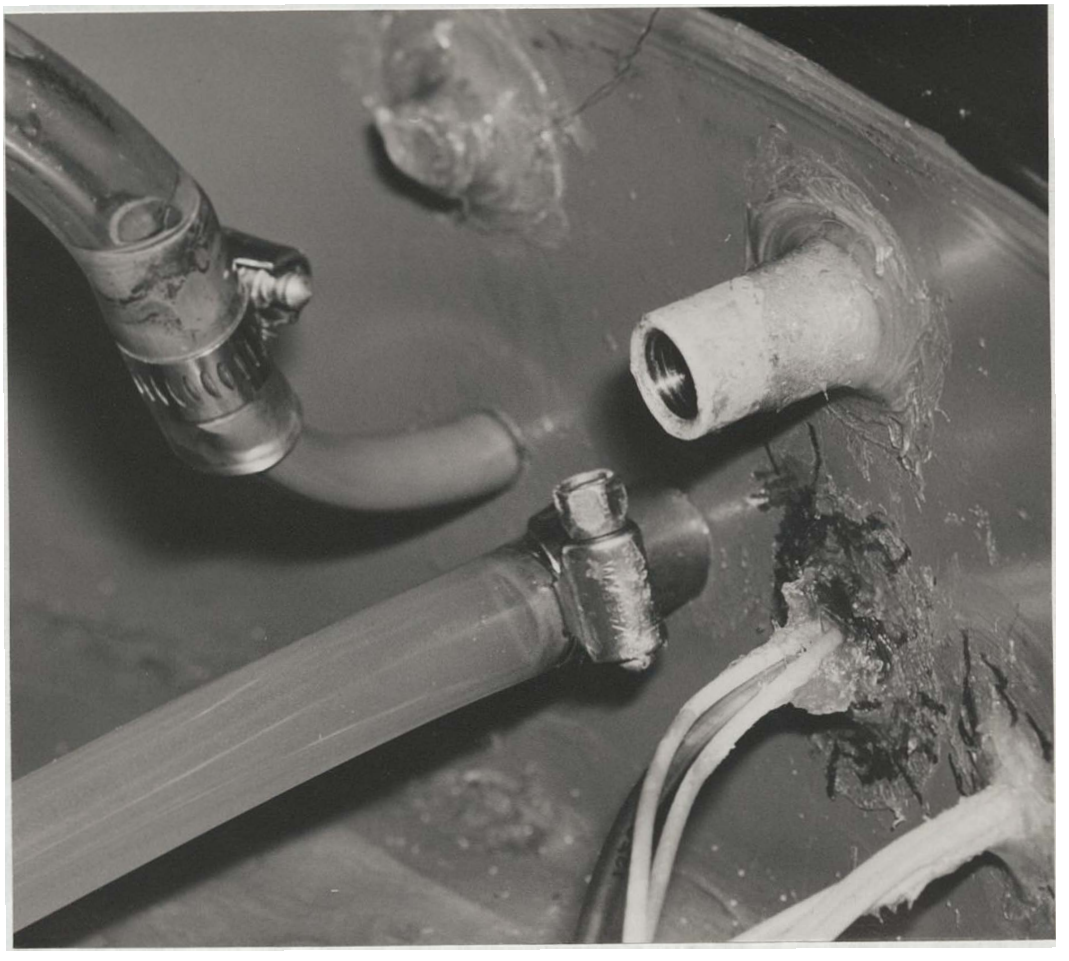

Fig. 5. Side-wall of assay chamber showing: one sample port, one acetylene gas entry port, two coolant hoses, cords of one environmental control thermocouple, two thermistors, and electrical power lines.

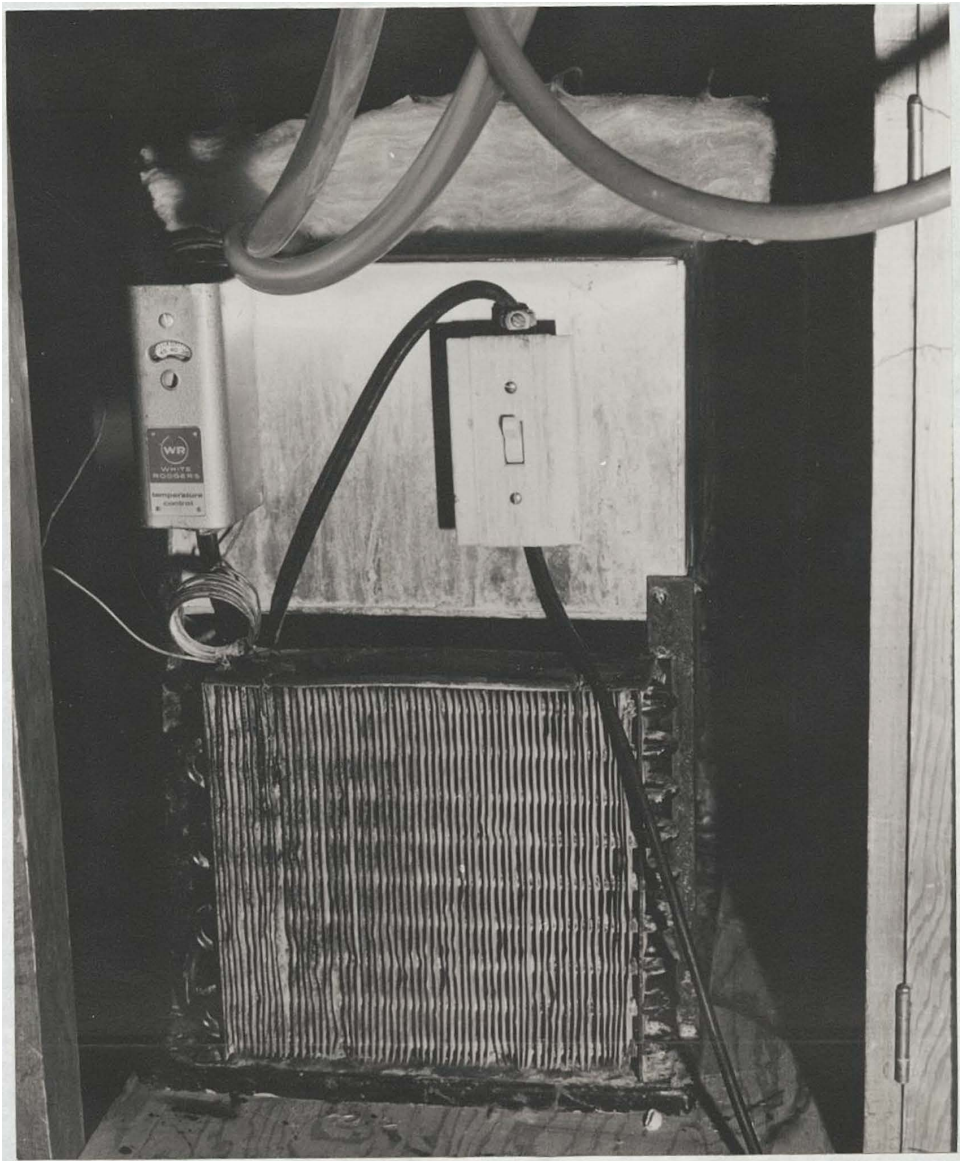

Fig. 6. Refrigeration unit.

Fig. 7. Coolant reservoir.

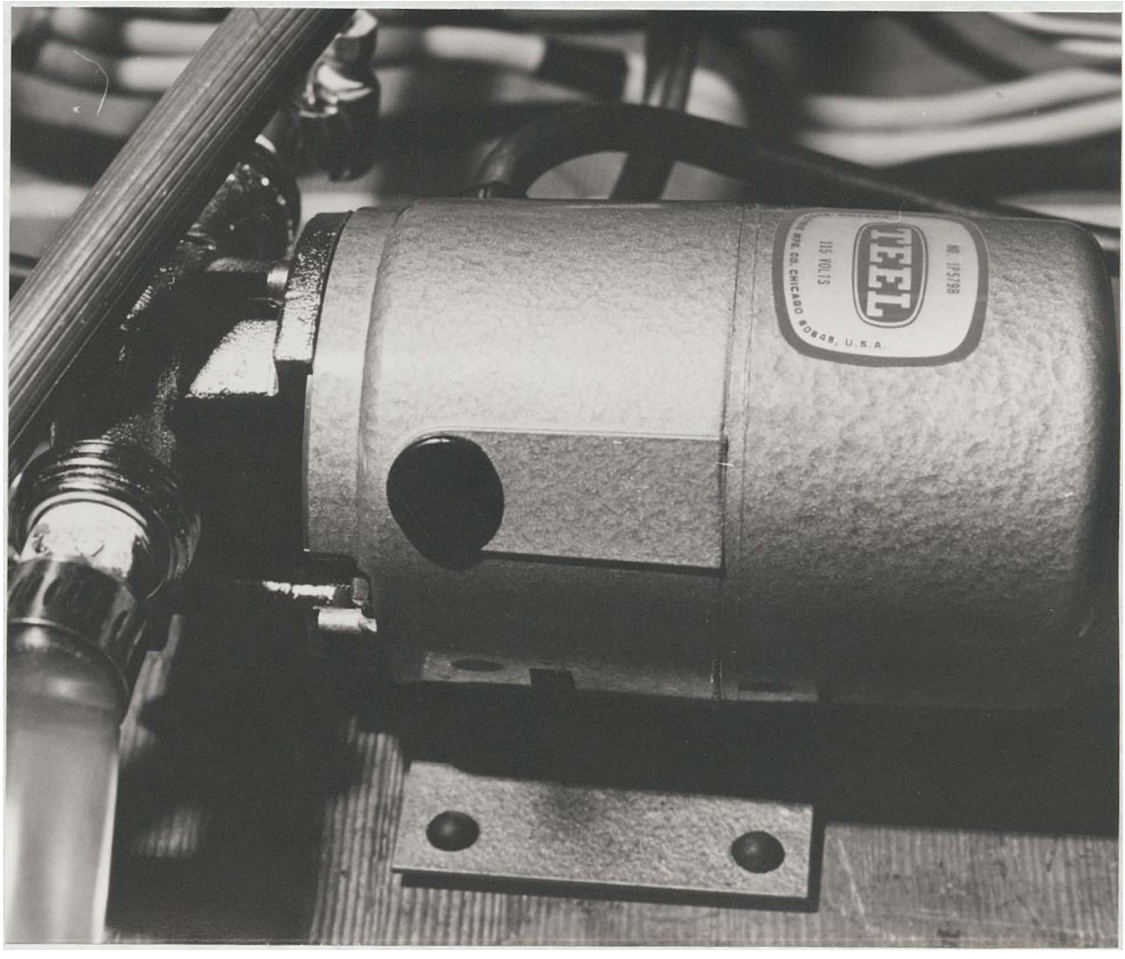

Fig. 8. Teel inline pump.

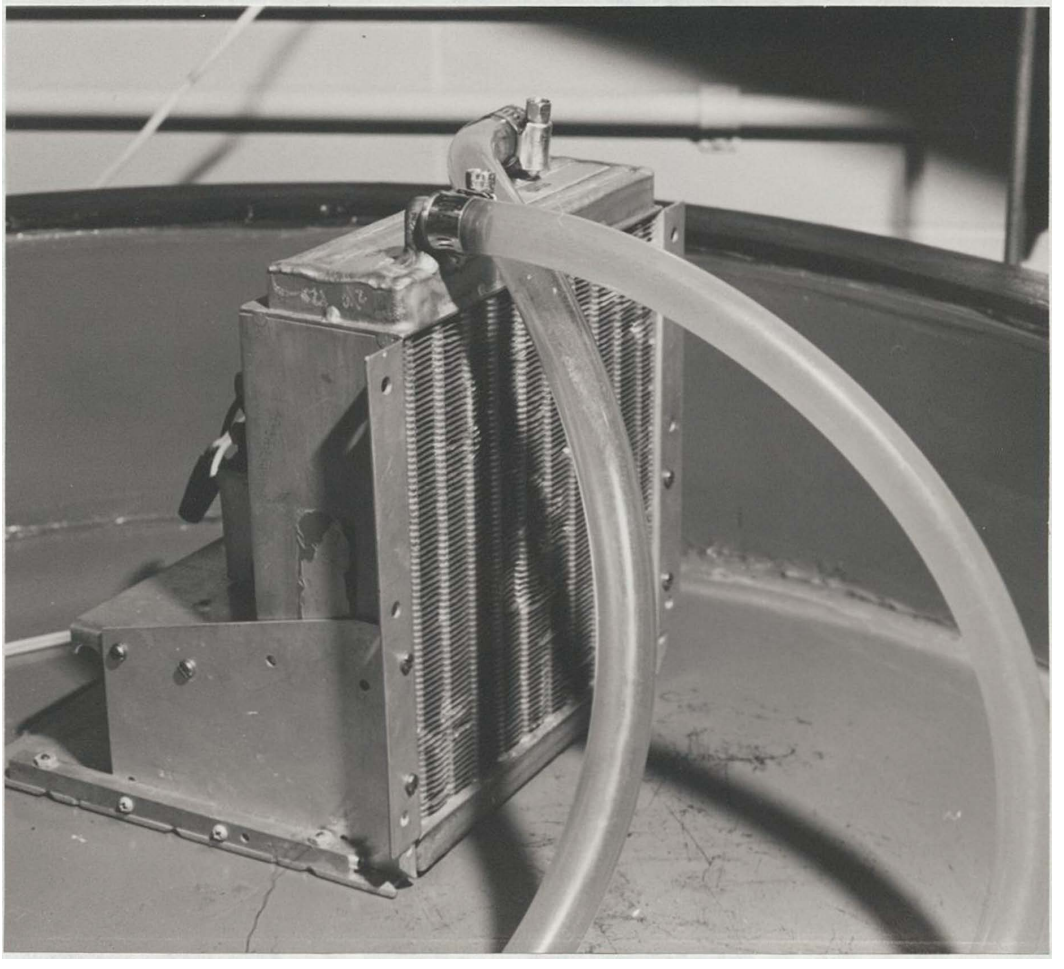

Fig. 9. Heat exchange unit.

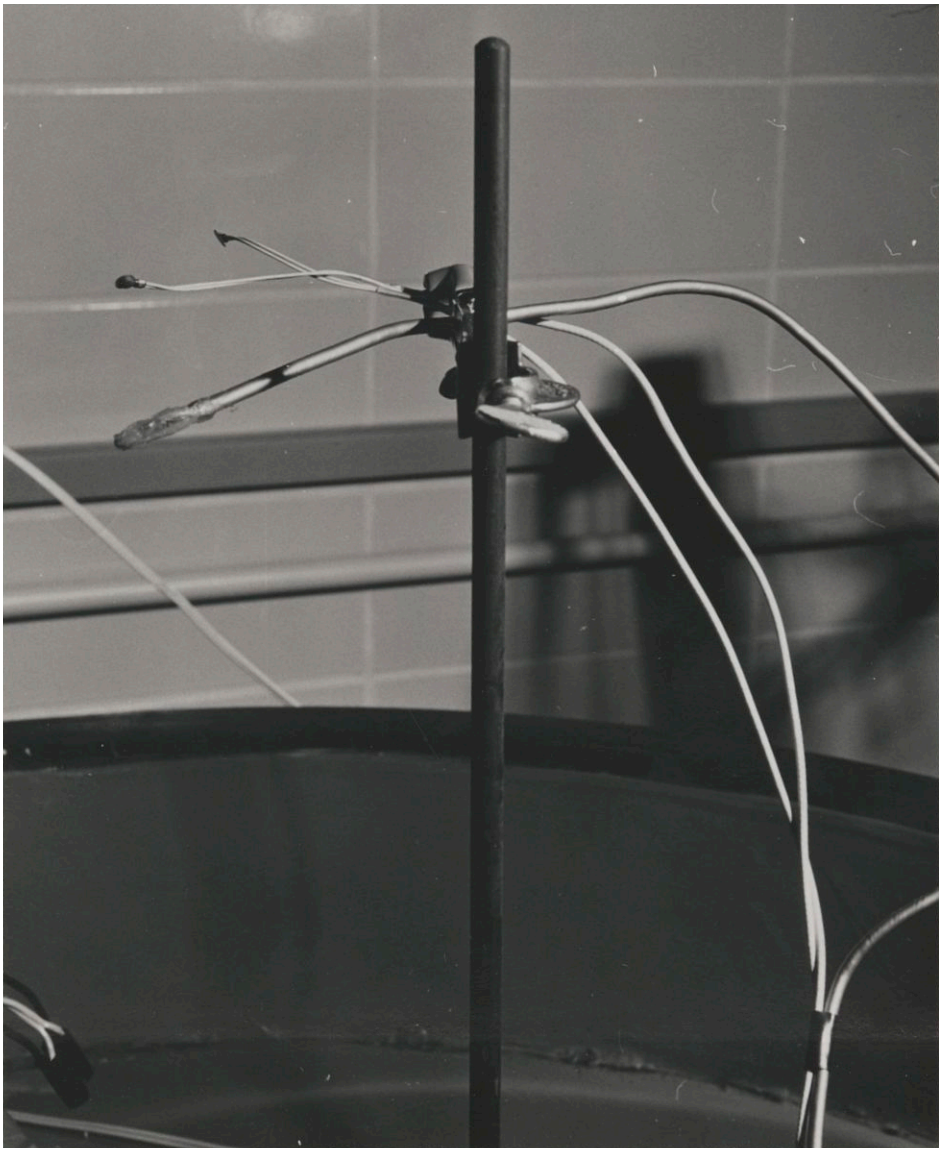

Fig. 10. Linear thermistors and thermocouple.

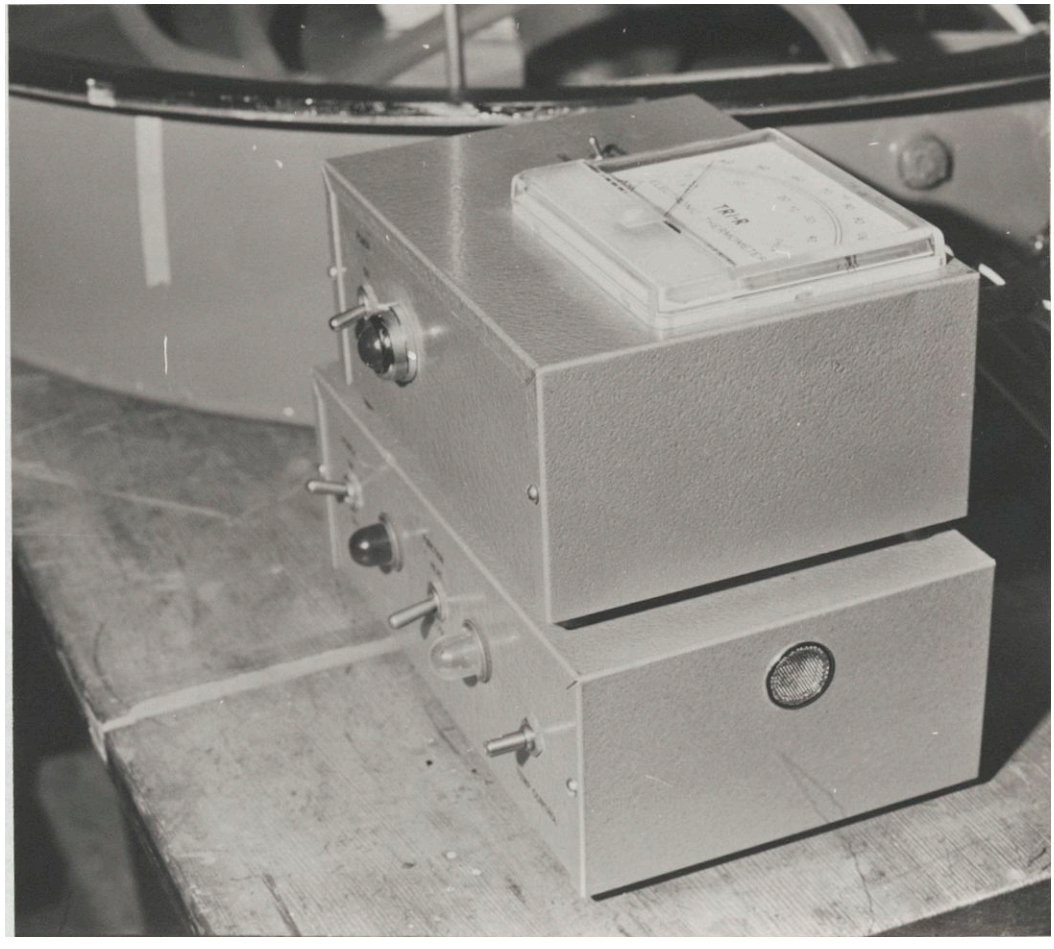

Fig. 11. TRI-R electric thermometer and environmental controller.

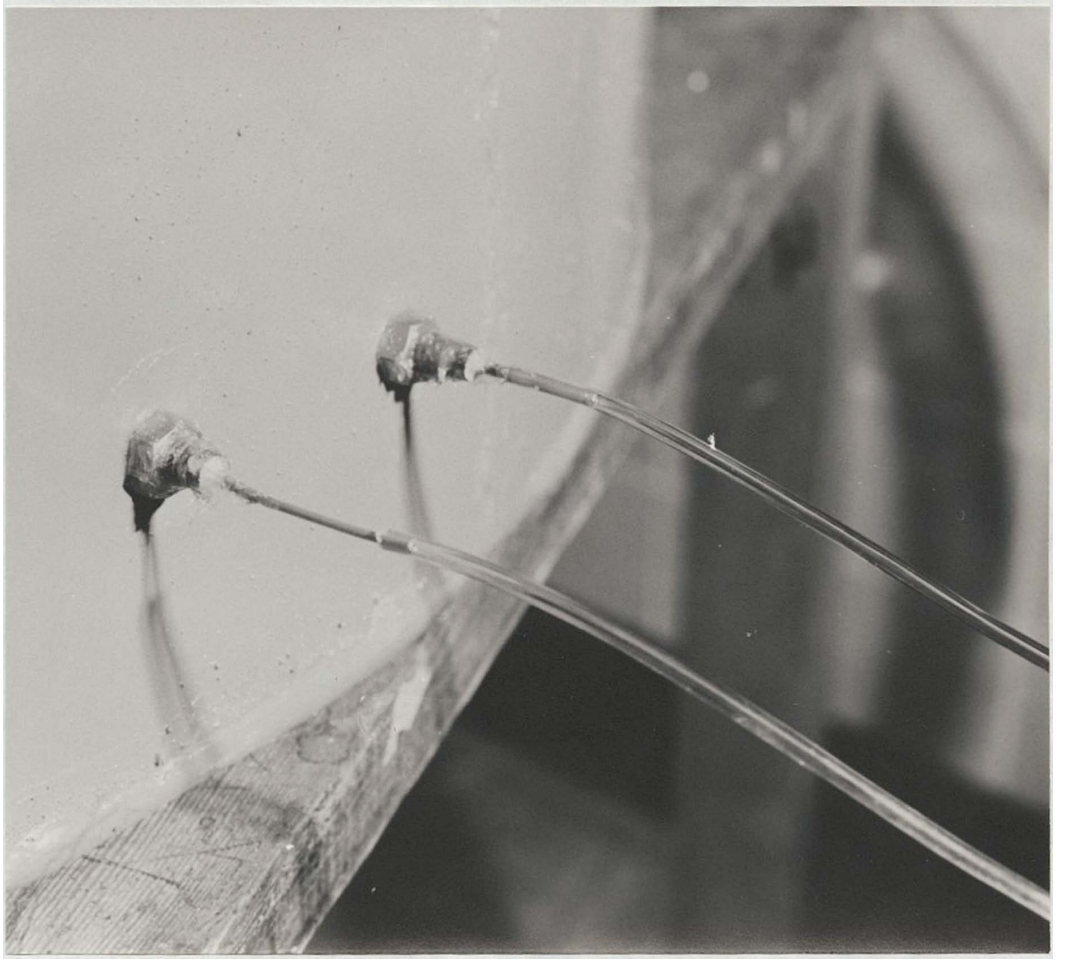

Fig. 12. Air ducts with plastic hoses leading to gas chromatograph sample valve.

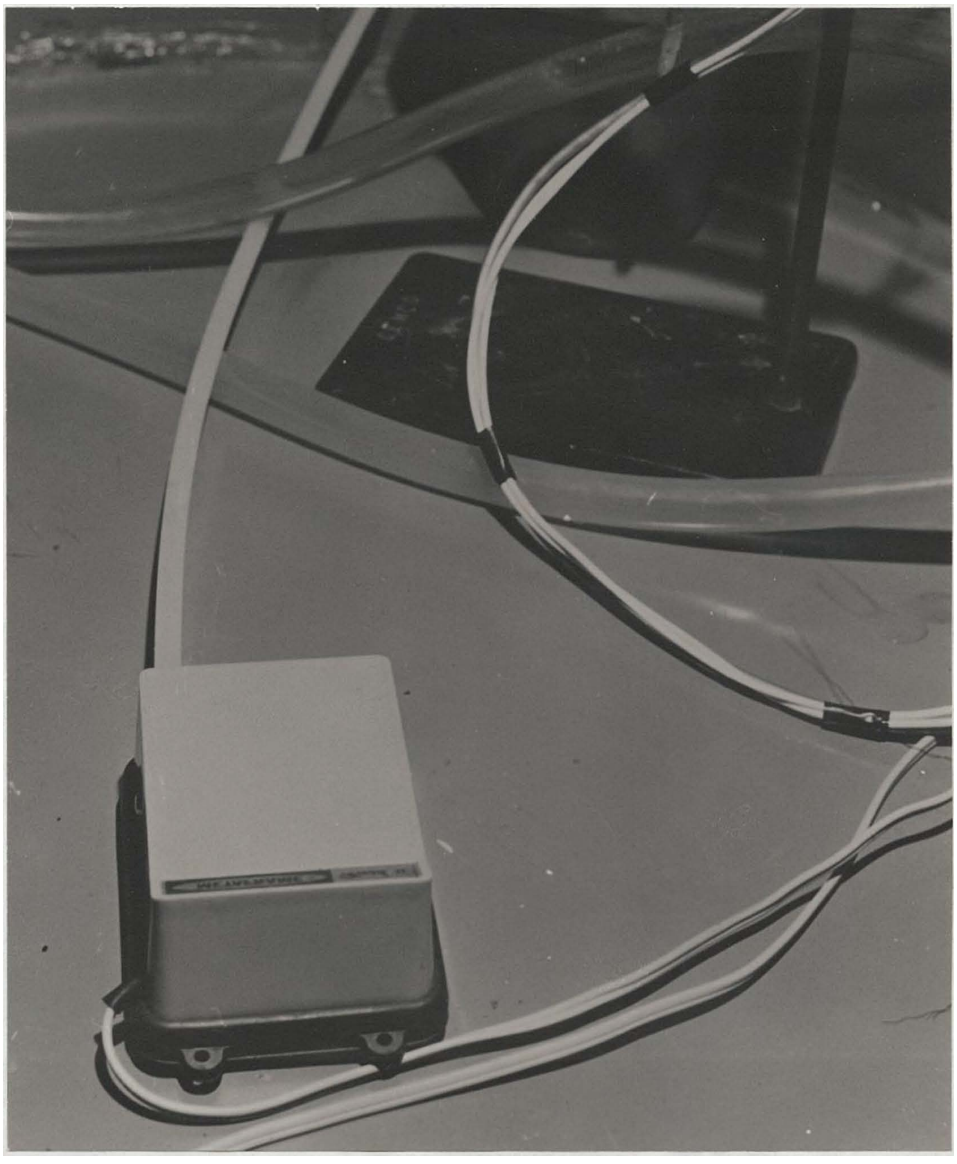

Fig. 13. Sample injection pump.

Calibration Results

The assay chamber is gas tight as shown by calibration results (see Table 1). The means of the areas under the gas chromatograph curves for the empty chamber do not differ significantly (P = 0.05) after 40 hours. This is important since the amount of ethylene gas produced during assay periods is minute (in some instances less than 4 ppm). Any loss of gases could markedly change quantitative estimates of fixation.

The bottled acetylene gas contained 1 ppm ethylene gas. This amount was then used as the zero point on the standard curve (see Figure 14) by which the amount of ethylene produced by plants was determined (Table 2). Results of plants without nodules show that soybeans did not produce endogenous ethylene that would increase the amount of background gas. Therefore any increase in ethylene concentration above this trace amount was considered to represent biological nitrogen fixation. In order to get usable curves representing ethylene production, the gas chromatograph was set to certain range and output values, i.e., range = 1.0; output = 256.0.

Another way to check for ethylene production by the experimental controls is by checking the concentration of acetylene gas at the beginning of the experimental period and after 40 hours have elapsed. Any decrease in acetylene indicates its reduction to ethylene since it has already been demonstrated that acetylene is not being lost via gas leaks. Thus two techniques were used to determine whether or not nitrogen fixation had taken place.

Experimental Results

The basic experimental objective is to quantitatively measure the amounts of nitrogen fixed utilizing the acetylene reduction assay for

nitrogen fixation. Table 2 reports the results for ethylene production in ppm. Understandably this assay method is not as sensitive as *in vitro* experiments for at least two reasons: (1) the environmental controls of this assay are not as exact, (2) and purified enzyme or cell extracts are used with *in vitro* experiments while the method developed in this study works with the whole plant for assaying acetylene reduction. This assay method was designed to approximate field conditions and in spite of any problems due to sampling procedures, background noise of the gas chromatograph, and ethylene contamination of the acetylene, the method is sensitive enough to detect as little as 1 ppm of ethylene gas as a result of acetylene reduction by experimental plants. One ppm is approximately equivalent to 0.5 pounds per acre of nitrogen fixed assuming a ratio of acetylene reduced: NH_3 formed of 3:1 (Hardy et al., 1971). The ppm of nitrogen reduced calculated from the above relationship (see Table 3) are in good agreement with results obtained by Hardy et al. in 1968. From data reported in Tables 2 and 4 it will be seen that the regression of nodule fresh weight to biomass of plant is nonsignificant. It thus appears that the physiological status of the plant is probably more important in relation to amount of nitrogen fixed than biomass of the entire plant. However nodule weight is correlated ($r = 0.7056$; $0.1 \quad P \quad 0.05$) with the amount of nitrogen fixed (Figure 15).

It is to be expected that nitrogen fixation would increase with increasing nodule weight. The relationship shown in Figure 15 would probably have been stronger if the nodules had been separated into size categories so that surface to volume effects could have been evaluated. In spite of this, the data seem precise enough to justify the use of this method for field studies of nitrogen fixation.

TABLE 1

RELATIVE VALUES FOR AREAS UNDER ACETYLENE GAS PEAKS

Treatment	20 hours			40 hours		
	n	$\bar{X}$	S	n	$\bar{X}$	S
Empty chamber	21	279	0.022	26	271	0.021
	64	274	0.037	41	270	0.020
Empty clay pot	50	276	0.020	51	270	0.010
	40	274	0.010			
Pot with dry soil	33	264	0.010	40	270	0.010
	46	263	0.012			
Pot with wet soil	20	268	0.009	31	270	0.010
	20	265	0.010			

TABLE 2

ETHYLENE PRODUCTION (ppm) BY SOYBEAN PLANTS

Plant	Period of Sampling (hours) from Initiation of Assay											
	1		4		20		30		40		70	
	$\bar{X}$	S	$\bar{X}$	S	$\bar{X}$	S	$\bar{X}$	S	$\bar{X}$	S	$\bar{X}$	S
1	5.17	0.002	4.87	0.001	4.58	0.001						
2	4.28	0.000	6.07	0.002	7.25	0.001						
3	4.28	0.001	4.28	0.000	10.83	0.001	13.81	0.000				
4	3.98	0.000	4.58	0.001	4.58	0.001	4.87	0.006				
5	4.87	0.001	4.58	0.000	4.58	0.001	6.36	0.003	8.45	0.002		
6	4.58	0.000	4.58	0.001	7.85	0.002	11.73	0.001	16.79	0.001	15.5	0.001
7	3.98	0.001	5.17	0.001	10.83	0.001	12.92	0.001				

TABLE 3

NITROGEN PRODUCED (ppm) BY SOYBEAN PLANTS AS CALCULATED FROM TABLE 2 (C_2H_4:NH_3 = 3:1)

Plant	Period of Sampling (hours) from Initiation of Assay					
	1	4	20	30	40	70
1	1.72	1.62	1.53			
2	1.43	2.02	2.42			
3	1.43	1.43	3.61	4.56		
4	1.33	1.53	1.53	1.62		
5	1.62	1.53	1.53	2.12	2.82	
6	1.53	1.53	2.62	3.91	5.60	5.2
7	1.32	1.72	3.61	4.31		

TABLE 4

BIOMASS DATA OF EXPERIMENTAL PLANTS

Plant	Dry weight entire plant in grams	Dry weight nodules only in grams	Fresh weight nodules only in grams	# nodules collected per pot	# plants nodulated per pot	# plants per sample pot
1	3.18	0.003	0.008	3	1	9
2	2.92	--	0.096	--	--	10
3	3.82	0.055	0.27	24	5	6
4	4.58	0.015	0.024	11	3	13
5	4.93	0.051	0.126	29	6	8
6	2.89	0.031	0.135	7	3	7
7	6.56	0.034	0.152	20	7	13

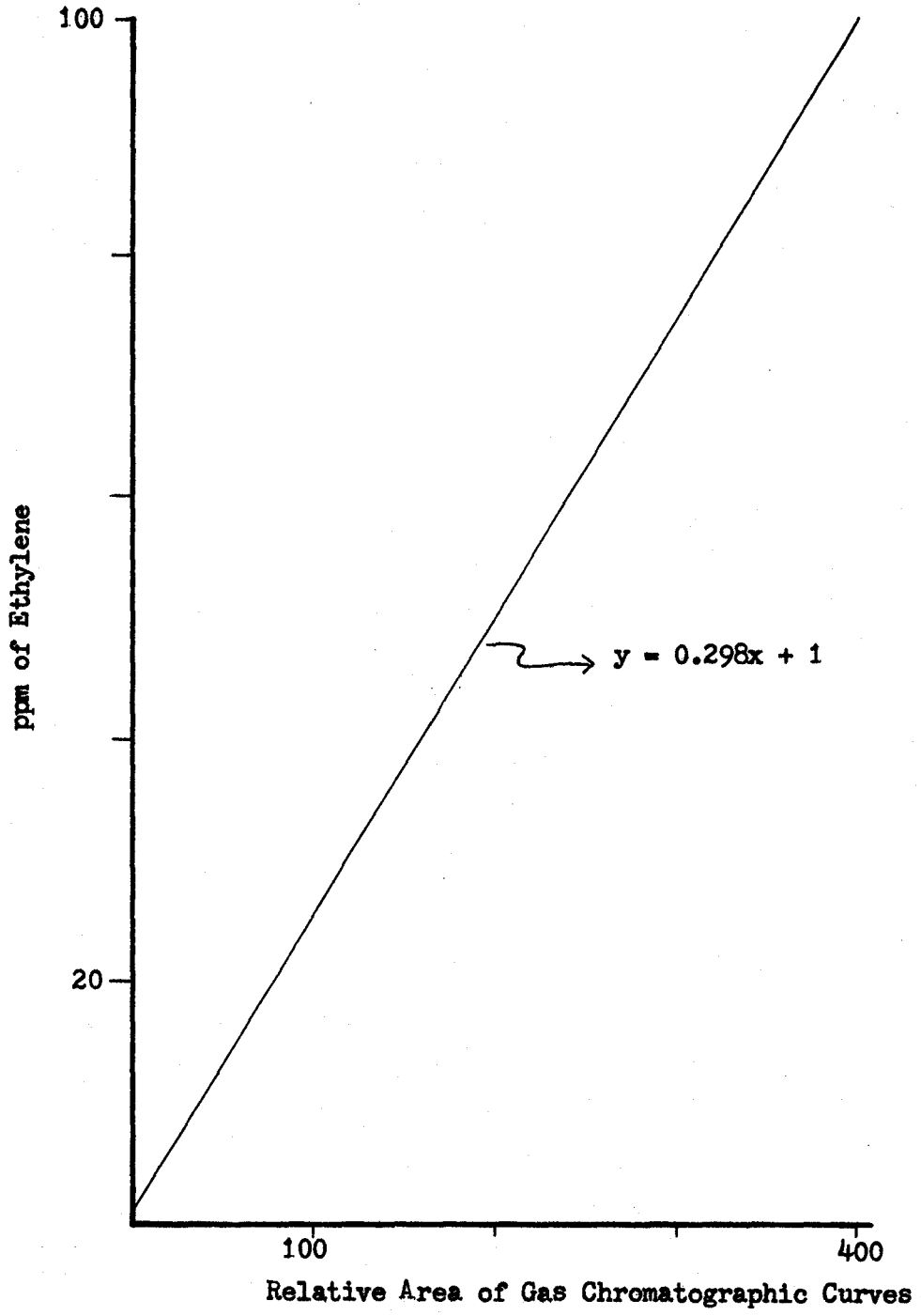

Fig. 14. Standard curve for determination of amount of ethylene fixed.

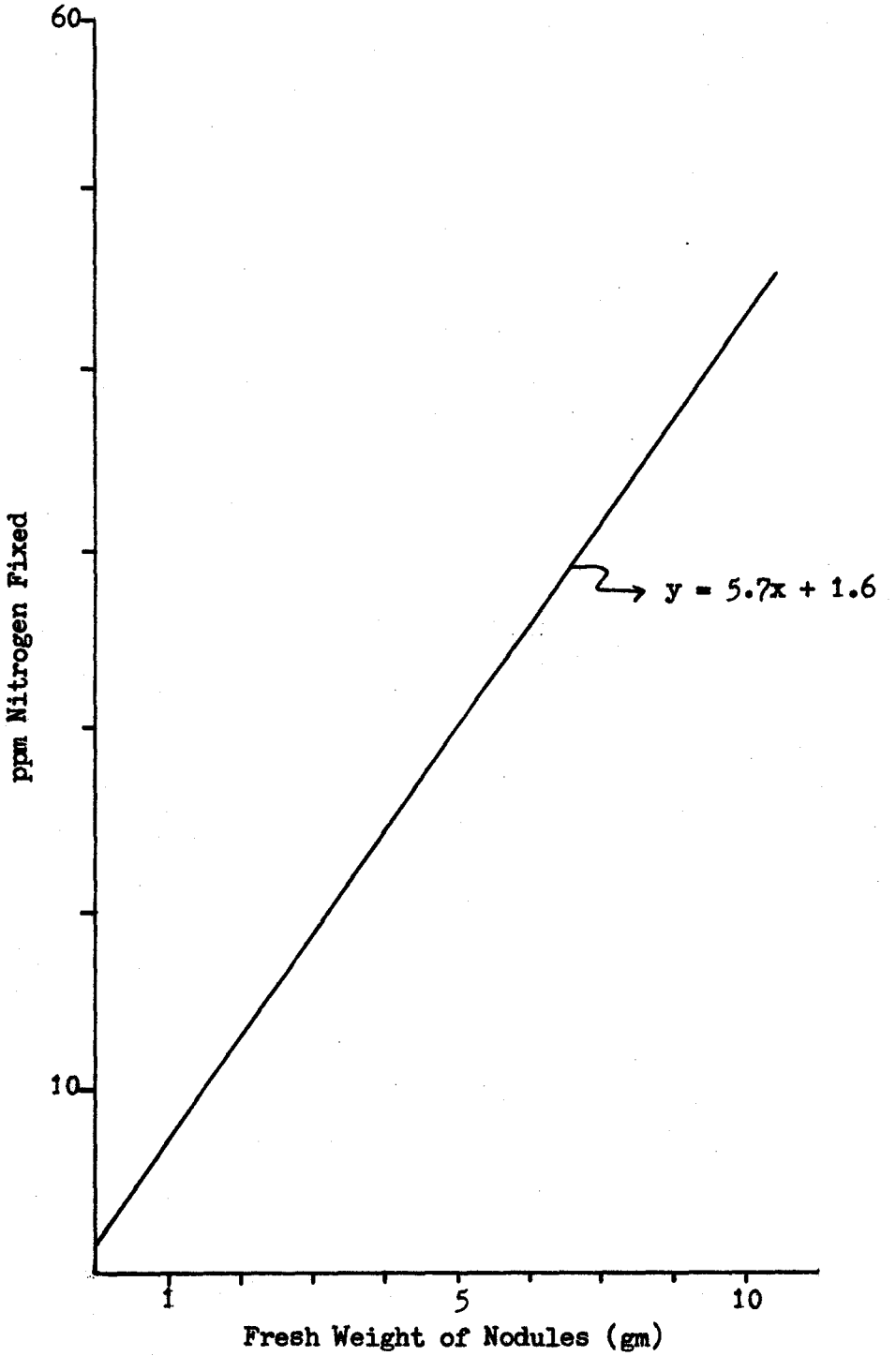

Fig. 15. Regression of nodule weight to amount of nitrogen fixed

SYSTEM EVALUATION AND SUMMARY

The assay system has been successfully used to quantify biological nitrogen fixation by intact legumes. The assay apparatus is unique, since it is the first system designed and successfully used to continuously and quantitatively monitor biological nitrogen fixation by intact plants utilizing the acetylene reduction technique. Other possible applications of the system are for analysis of photosynthetic and respiration rate studies of above ground foliage and soil biota. Soils might also be treated with various gases (e.g., SO_2, N_2O, etc.) to test their effect on soil biota.

Under normal operating procedures, there are four possible sources for the appearance of ethylene gas within the assay chamber environment: (1) from the room air, (2) from the bottled acetylene gas used for the acetylene reduction assay, (3) from free-living nitrogen fixers in the culture medium, and (4) from rhizobial activity of nodulated legumes. Controls were run that eliminated enrichment of ethylene gas concentrations within the assay chamber from the room air. Background amounts of ethylene were found in the bottled acetylene. This background ethylene proved useful for detection of biological nitrogen fixation. Any increase in concentration of ethylene gas above the background level was taken as an indication that biological nitrogen fixation occurred. Soil samples which were maintained and watered in the same manner as those in which the legumes were grown were tested for possible ethylene production by free-living bacteria or blue-green algae. No

enrichment of ethylene concentrations occurred in the presence of these soil samples. However, when potted legumes were incubated in the assay chamber with 1200 milliliters of acetylene gas, enrichment of ethylene gas concentrations within the chamber environment was detected by gas chromatographic analysis.

Therefore, the assay system which resulted from this research has fulfilled the objectives of supplying quantitative data on biological nitrogen fixation by intact plants. Since the environmental factors of light and temperature can be regulated, it should be possible in future studies to evaluate the effects of temperature and light intensity on fixation rates. The effects of other factors such as biomass, leaf surface, and amounts of above-ground foliage could also be evaluated for their effect on biological nitrogen fixation rates.

LITERATURE CITED

Allen, E. K. and O. N. Allen. 1965. Nonleguminous plant symbiosis in Microbiology and Soil Fertility. Oregon State Univ. Press. pp. 77-106.

Allison, F. E. 1955. The enigma of soil nitrogen balance sheets. Adv. Agron. 7:213-250.

Allison, F. E., S. R. Hoover, and F. W. Minor. 1942. Biochemical nitrogen fixation studies IV. Experiments with excised legume nodules. Bot. Gaz. 104:63.

Aprison, M. H., and R. H. Burris. 1952. Time course of fixation of N_2 by excised soybean nodules. Sci. 115:264.

Atwater, W. O. 1885. On the acquisition of atmospheric nitrogen by plants. Amer. Chem. Jour. 6:365-388.

Atwater, W. O. 1886. III. On the liberation of nitrogen from its compounds and the acquisition of atmospheric nitrogen by plants. Amer. Chem. Jour. 8:398-420.

Ballentine, R. 1957. Determination of total nitrogen and ammonia in Methods of Enzymology. (Colowick and Kaplan, eds.) 3:984-985. Academic Press, New York.

Bergersen, F. J. 1970. The quantitative relationship between nitrogen fixation and the acetylene-reduction assay. Aust. Jour. Biol. Sci. 23:1015-25.

Bergersen, F. J. and A. B. Costn. 1964. Root nodules on Podocarpus lawrencei and their ecological significance. Aust. Jour. Biol. Sci. 17:44-48.

Bond, G. 1959. The incidence and importance of biological fixation of nitrogen. Adv. Sci. 15:382-386.

Bradstreet, R. B. 1940. A review of the Kjeldahl determinations of organic nitrogen. Chem. Revs. 27:331-350.

Burris, R. H. 1941. Failure of barley to fix N^{15}. Sci. 93:114-115.

Burris, R. H. 1965. Nitrogen fixation in Plant Biochemistry. (Bonner and Varner, eds.) pp. 961-979. Academic Press, New York.

Burris, R. H. 1974. Biological nitrogen fixation, 1924-1974. Plant Physiol. 54:443-449.

Burris, R. H. and P. W. Wilson. 1957. Methods for measurement of nitrogen fixation in Methods of Enzymology. (Colowick and Kaplan, eds.) 4:355-367. Academic Press, New York.

Burris, R. H., F. J. Eppling, H. B. Wahling and P. W. Wilson. 1942. Studies of biological nitrogen fixation with isotopic nitrogen. Soil Sci. Soc. Amer. Proc. 7:258-262.

Burris, R. H., F. J. Eppling, H. B. Wahling and P. W. Wilson. 1943. Detection of biological nitrogen fixation with isotopic nitrogen. Jour. Biol. Chem. 148:349.

Campbell, N. E. R. 1967. In Colloqium of biological nitrogen fixation (W. S. Silver, ed.) Sci. 157:100-102.

Campbell, N. E. R., R. Dular, H. Less, and K. G. Standing. 1967. The production of N_2^{13} by 50 mev proton for use in biological nitrogen fixation. Can. Jour. Microbiol. 13:587-599.

Carnahan, J. E., L. E. Mortenson, H. F. Mower, and J. E. Castle. 1960. Nitrogen fixation in cell-free extracts of Clostridium pasteurianum. Biochem. Biophys. Acta. 44:520-535.

Conway, E. J. 1960. Microdiffusion analysis and volumetric error. Cosby, Lockwood and Son, Ltd., London. 467 pp.

Delwiche, C. C., and P. L. Steyn. 1970. Nitrogen isotope fractionation in soils and microbial reactions. Environmental Sci. & Tech. 4:929-935.

Dhar, N. R. 1959. Phosphates and organic substances in nitrogen deficiency. Nature. 183:513-514.

Dilworth, M. J. 1966. Acetylene reduction by nitrogen-fixing preparations from Clostridium pasteurianum. Biochem. Biophys. Acta. 127:285-294.

Donald, C. M. 1960. The impact of cheap nitrogen. Jour. Aust. Inst. Agric. Sci. 26(4). 319-338.

Ehrlich, P. R. 1968. The population bomb. Ballentine Books Inc. 201 pp.

Farnsworth, R. B. and M. W. Hammond. 1968. Root nodule and isolation of endophyte on Artemisia ludoviciana. Utah Acad. Proc. 45(1): 182-188.

Hallet, L. T. 1949. The analysts column. Anal. Chem. 21:17A.

Hardy, R. W. F. and R. C. Burns. 1968. Biological nitrogen fixation, in Ann. Rev. of Biochem. 37:331-58.

Hardy, R. W. F. and E. Knight Jr. 1967. ATP-dependent reduction of azide and HCN by N_2-fixing enzymes of Azotobacter vinelandii and

Clostridium pasteurianum. Biochem. Biophys. Acta. 139:69-90.

Hardy, R. W. F. and E. Knight Jr. 1968. Biochemistry and postulated mechanisms of nitrogen fixation in *Progress in Phytochemistry*. (Reinhold and Liuschitz, eds.), pp. 407-489. Inter. Sci. Pub., New York.

Hardy, R. W. F., E. Knight, and E. K. Jackson. 1967. Substrate versatility of nitrogenase. Bacteriol. Proc. pp. 112.

Hardy, R. W. F., R. D. Holsten, E. K. Jackson, and R. C. Burns. 1968. The acetylene-ethylene assay for N_2 fixation: laboratory and field evaluation. Plant Physiol. 43:1185-1207.

Hardy, R. W. F., R. C. Burns, R. R. Herbert, R. D. Holsten and E. K. Jackson. 1971. Biological nitrogen fixation a key to world protein, in biological nitrogen fixation in natural and agricultural habitats (Lie and Mulder, eds.) Pl. Soil Special. Vol: 561-590.

Hawker, L. E. and J. Fraymouth. 1951. A reinvestigation of the root nodules of species of *Elaeagnus*, *Hippoplae*, *Alnus*, and *Myrica*, with special reference to the morphology and life histories of the causative organisms. Jour. Gen. Microbiol. 5:369-386.

Hellriegel, H. and H. Wilfarth. 1888. Untersuchungen über die Stickstaffnahrung der Gramineen und Leguminosen. Beilageheft zu der Ztschr. Ver. Rübenzucker-Industrie Deutschen Reichs. 234 pp.

Hiller, A., J. Plazin and D. D. Van Slyke. 1948. A study of conditions for Kjeldahl determination of nitrogen in proteins. Jour. Biol. Chem. 176:1401-1420.

Holiday, D. A. 1971. New nitrogen source: fixation by non-legumes might help many soils. Crops and Soils. pp. 15-18.

Hurwitz, C. and P. W. Wilson. 1940. Direct estimation of biological nitrogen fixation. A gasometric method. Ind. Eng. Chem. Anal. Ed. 12:31-33.

Kjeldahl, J. 1883. Neue Methode zur Bestimmung des stickstoffs in Organischem Korpern. Z. Anal. Chem. 22:366-382.

Klucas, R. V., B. Koch, S. A. Russell, and H. J. Evans. 1968. Purification and some properties of the nitrogenase from soybean nodules. Plant Physiol. 43:1906-1912.

Koch, B. and J. H. Evans. 1966. Reduction of acetylene to ethylene by soybean root nodules. Plant Physiol. 41:1748-1750.

Koch, B., H. J. Evans, and S. Russell. 1967a. Reduction of acetylene and nitrogen gas by breis and cell-free extracts of soybean root nodules. Plant Physiol. 42:466-468.

Koch, B., H. J. Evans, and S. Russell. 1967b. Properties of the nitrogenase system in cell-free extracts of bacteroids from soybean root nodules. Proc. Natl. Acad. Sci. U.S. 58:1343-1350.

Machlis, L. and J. G. Torrey. 1956. Plants in action. p. 44. W. H. Freeman and Co., San Francisco.

McKee, H. S. 1962. Nitrogen metabolism in plants. Claredon Press, Oxford. 728 pp.

Mortenson, L. E. 1961. A simple method for measuring nitrogen fixation by cell-free enzyme preparations of Clostridium pasteurianum. Anal. Biochem. 2:216-220.

Moustafa, E. 1969. Cold lability of the nitrogen fixation system in excised leguminous nodules. Phytochem. 8:993-995.

Moustafa, E. and L. E. Mortenson. 1967. Acetylene reduction by nitrogen fixing extracts of Clostridium pasteurianum: ATP requirements and inhibition by ADP. Nature. 216:1241-1242.

Moustafa, E., R. Ball, and T. R. O. Field. 1969. The use of acetylene reduction to study the effects of nitrogen fertilizer and defoliation on fixation by field-grown white clover. New Zealand Jour. Agri. Research. 12:691.

New Republic. 1965. Editorial: Fighting world famine. 153:8.

Nicholas, D. J. D., D. J. Silvester, and J. F. Fowler. 1961. Use of radioactive nitrogen in studying nitrogen fixation in bacterial cells and their extracts. Nature. 189:634-636.

Paddock, W. P. 1958. Famine - 1975! Little, Brown and Co., Boston. 276 pp.

Postgate, John. 1970. Nitrogen fixation. Trop. Sci. 12(3):223-229.

Roughley, R. J. and P. J. Dart. 1969. Reduction of acetylene by nodules of Trifolium subterraneum as affected by root temperature. Rhizobium strain and host cultivar. Arch. Mikrobiol. 69:171-179.

Russell, S. A. and H. J. Evans. 1966. The nitrogen-fixing capacity of Ceanothus velutinus. Forest Sci. 12(2):164-169.

Rychert, R. C. and J. Skujins. 1973. Microbiol. activity in acid soils. Utah Sci. 34(3):96-98.

Salisbury, F. B. and C. Ross. 1969. Plant physiology. pp. 330-348. Wadsworth Pub. Co. Inc. Belmont, Calif.

Schneider, A. 1894. Mutualistic symbiosis of algae and bacteria with Cycas revoluta. Bot. Gaz. 19:25-32.

Schollhorn, R. and R. H. Burris. 1967. Acetylene as a competitive inhibitor of N_2 fixation. Proc. Natl. Acad. Sci. U. S. 58: 213-216.

Schwinghamer, E. H., H. J. Evans, and M. D. Dawson. 1970. Evaluation of effectiveness in mutant strains of Rhizobium by acetylene reduction relative to other criteria of N_2 fixation. Plant and Soil. 33:192-212.

Silver, W. S. 1967. Biological nitrogen fixation. Sci. 157:100-102.

Silver, W. S., Y. M. Centifanto, and D. J. D. Nicholas. 1963. Nitrogen fixation by the leaf nodule endophyte of Psychotria bacteriophila. Nature. 199:396-397.

Sprent, J. 1969. Prolonged reduction of acetylene by detached soybean nodules. Planta. 88:372-375.

Stewart, W. D. P. 1967. Nitrogen-fixing plants. Sci. 158:1426-1432.

Stewart, W. D. P., G. P. Fitzgerald and R. H. Burris. 1967. In situ studies of nitrogen fixation with the acetylene reduction technique. Sci. 158:536.

Vickery, H. B. 1946. A modification of the Kjeldahl analysis. Yale Jour. Biol. and Med. 18:473.

Virtanen, A. I. 1939. Mechanisms of symbiotic nitrogen fixation by leguminous plants. Trans. Third Comm. Internat. Soc. Soil Sci. A:4-19.

Waksman, S. A. 1927. Principles of soil microbiology. Baltimore, The Williams and Wilkins Co.

Wilson, P. W. 1939. in Nord, F. F., and Weidenhagen, R., Ergebnisse der Enzymforschung, Leipzig, 8:13.

Wilson, P. W. 1940. The biochemistry of symbiotic nitrogen fixation. Univ. of Wisconsin Press, Madison, Wisconsin. 302 pp.

Wilson P. W. 1951. Biological nitrogen fixation in Bacteria Physiology (Workman and Wilson, eds.) pp. 471-499. Academic Press, New York.

Wilson, P. W. 1963. Biological nitrogen fixation--early American style. Bacteriological Revs. 27:405-416.

Wood, B. W. 1971. Response of Canada Milkvetch (Astragalus canadensis var. mortonii (Nutt.) Wats.) to Range and Forest Improvement Practices in Northeastern Oregon. (Corvallis, Oregon). Unpublished Ph.D. Dissertation, Oregon State University.

AN ASSAY CHAMBER FOR QUANTITATIVE ANALYSIS OF NITROGEN FIXATION USING INTACT PLANTS

Thomas A. Leslie, Jr.

Department of Botany and Range Science

M.S. Degree, April 1975

ABSTRACT

A unique assay system for quantitative analysis of biological nitrogen fixation by intact plants has been constructed and successfully tested. With the system, it has been possible to detect acetylene reduction by intact legumes *in situ* under laboratory conditions. This assay method is designed to approximate field conditions and in spite of possible procedural errors in sampling, background noise of the gas chromatograph and ethylene contamination of the acetylene gas used in the assay, this method is sensitive enough to detect as little as 1 ppm enrichment of ethylene gas as a result of acetylene reduction by intact soybean plants. The determinations of nitrogen fixed are in good agreement with results obtained by other researchers who have used the acetylene reduction technique in *in vitro* studies.