EFFECT OF TEMPERATURE AND ADDED GLUCOSE ON NITROGEN FIXATION IN SOIL AS MEASURED BY ACETYLENE REDUCTION AND ^{15}N TRACER METHOD

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ABSTRACT

The effects of energy addition (glucose) and incubation temperature upon non-symbiotic N-fixation were studied by ^{15}N tracer method and acetylene reduction method on a Manitoba soil. Incubation vessels for N-fixation study using both techniques were developed and used in this study.

At temperatures, 15°C, 25°C, and 35°C, there was very little N-fixation in the absence of an added energy source. The addition of glucose at rates of 0.04% and 0.2% increased N-fixation slightly when compared with the control sample. Glucose at 1% greatly increased N-fixation at all temperatures. An increase in the glucose level to 5% resulted in more fixation than with 1% glucose at 25°C. At 35°C fixation with 5% glucose was slightly less than with 1% glucose, and at 15°C it was considerably lower.

The relative values obtained by the ^{15}N tracer method and the C_2H_2 reduction method are in good agreement, but the magnitude of N-fixation calculated from C_2H4 produced, was consistently lower than that of the ^{15}N tracer method. The data suggest that the conversion factor for moles of nitrogen fixed per mole of acetylene reduced for this particular soil sample is 4/3 rather than 1/3.

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INTRODUCTION

Nitrogen fixation is carried on non-symbiotically by a wide variety of free living soil microorganisms. These organisms are typified by Clostridium spp., and Azotobacter spp. Pseudomonas also contributes fixed nitrogen in the Canadian Prairie soils. The amount of combined nitrogen produced varies with temperature, richness of the soil and many other physical and biological factors. There has been little agreement as to how much nitrogen they actually fix and hence their importance to agriculture. This has been due to the difficulty of measuring accurately the amount of nitrogen being fixed.

It was not until 1941 that Burris and Miller introduced the application of ^{15}N to the study of biological nitrogen fixation. This method consists of incubating a soil in an atmosphere in which the nitrogen gas present is enriched with the stable isotope ^{15}N. The soil is analyzed to determine the amount of this isotope incorporated into the bacterial cell material and thus the organic material of the soil. Therefore, it provides an accurate estimation of fixation which has taken place. This method is expensive and time consuming. In 1967 the acetylene reduction method was proposed as a means to determine N-fixation by Hardy and Knight. This method is based on the fact that the enzyme, nitrogenase, which is responsible for the reduction of nitrogen in the fixation process is also capable of reducing acetylene to ethylene. Thus the assay of the enzyme activity is actually the measure of the nitrogen fixing activity. This method is sensitive, simple, less expensive than ^{15}N tracer method and not as time consuming.

The aim of this investigation was to determine the nitrogen fixing capacity of soil at different temperatures with different levels of glucose by the ^{15}N tracer method and the C_2H_2 reduction method, and also the suitability of these methods for measuring the N-fixation in soil.

LITERATURE REVIEW

Nitrogen fixation is a process in which atmospheric nitrogen is reduced to ammonia-nitrogen and is assimilated by microorganisms. The microorganisms mediating this process may be aerobic or anaerobic, heterotrophic or photoautotrophic, free living, or associated with plants such as legumes. Nitrogen fixation by free living microorganisms is the only concern here.

In 1862 Jodin(35), for the first time, reported on experiments with "mycodermes" which he considered to be able to metabolize elemental nitrogen. In 1888, Schloesing(55) failed to confirm Jodin's statement. In 1895, Winogradsky isolated the anaerobic nitrogen-fixing bacterium, Clostridium pasteurianum(60), and in 1901 Beijerinck isolated two species of Azotobacter which fixed nitrogen(4). The intensive studies that followed resulted in the isolation of other species of the two genera that fixed nitrogen. In the subsequent years, blue-green algae such as genera of Anabaena and Nostoc(24), genus Beijerinckia which resembles Azotobacter(22), genus Derxia(33), and many other nitrogen fixing micro-organisms were isolated and confirmed to have nitrogen fixing ability.

Physiological characteristics of some non-symbiotic N-fixers

A. The Azotobacteraceae.

This family comprises the nitrogen-fixing genera Azotobacter, Beijerinckia, and Derxia. They are gram-negative, strictly aerobic, and are heterotrophic rods.

a. Azotobacter spp.

The genus Azotobacter has world-wide distribution and it occurs

most commonly in soil. Fixation by Azotobacter is best under slightly acid to slightly alkaline conditions, pH 6-7.8, and is rapidly inhibited below pH 6(13). The optimum temperature for fixation and growth is approximately 25-28°C. Although strictly aerobic, fixation by Azotobacter may be inhibited at high partial pressure of oxygen(49,51,58). Schmidt-Lorenz and Rippel-Baldes(58) showed that A. chroococcum fixed 18-20 mg of nitrogen per gram of consumed glucose at 0.02-0.04 atmosphere oxygen, and its efficiency declined gradually to 10-11 mg of nitrogen per gram of consumed glucose at 0.4 atmosphere. At higher oxygen partial pressures, nitrogen fixation is inhibited completely. When the partial pressure of oxygen was 0.20 atmosphere, Parker(49) found 7-8 mg nitrogen fixed per gram sucrose added. Therefore, the nitrogen fixation ability of Azotobacter is in the order of 7-20 mg nitrogen per gram of sugar consumed at natural state.

b. Beijerinckia

This genus differs markedly from Azotobacter in its ability to grow within the pH range of 3-9. Its efficiency of nitrogen fixation is high and sometimes reaches 20-22 mg per gram of consumed sugar. After extensive study of 392 samples from different countries, such as Europe, Africa, Asia, Australia, North and South America, Becking concluded that the occurrence of Beijerinckia is limited to tropical soils(3).

c. Derxia

The genus Derxia has a very high fixation efficiency - up to 25 mg per gram of sugar consumed. Fixation occurs in the pH range 5-9 with an optimum temperature between 25°C and 35°C. This genus is found mostly in tropical soils(33).

B. The Bacillaceae

This family comprises the nitrogen-fixing genus Clostridium, all species of which are strict anaerobes, and the genus Bacillus all species of which are facultative anaerobes. Both genera are of gram-positive, spore-forming rods.

a. Clostridium spp.

The species involved in nitrogen fixation are C. butyricum, C. pasteurianum, and C. kluyven. These species have world-wide distribution. The optimum temperature for fixation is approximately 25°C. Its nitrogen fixing efficiency is in the order of 2-3 mg nitrogen fixed per gram of carbohydrate consumed(34), although C. butyricum has been reported to fix 27 mg of nitrogen per gram of carbohydrate(49). Nitrogen fixation is best at neutral pH.

b. Bacillus spp.

The nitrogen fixing ability of the species B. polymyxa was confirmed by Grau and Wilson in 1962(26). It grows at temperatures ranging from 25-37°C, and fixation is inhibited completely by as little as one percent oxygen.

C. Other nitrogen fixing microorganisms.

The genus Pseudomonas, first demonstrated to fix nitrogen by Anderson(2) in 1955, can fix nitrogen under both aerobic or anaerobic conditions. Paul and Newton(52) demonstrated that this organism fixed 104 mg of nitrogen per gram of mannitol consumed, and it can fix nitrogen at temperatures as low as 8°C.

Aerobacter aerogenes, a facultative anaerobe, was found to fix nitrogen by Hamilton and Wilson(31), and Pengra and Wilson(53).

Jensen(34), in 1956, found that Aerobacter aerogenes was able to fix approximately 4.5 mg nitrogen per gram of added glucose.

An experiment carried out by Proctor and Wilson(54) showed that all strains of Achromobacter fix nitrogen. One strain of Achromobacter fixes nitrogen anaerobically and the remainder fix nitrogen aerobically.

Abundance of nitrogen fixers in soil.

In the natural habitat in soil, the nitrogen fixing activity of the nitrogen-fixers varies a good deal depending on aeration, pH, available energy, moisture content and other environmental factors.

The microbial count for Azotobacter in soil is very low. In 1932, Ziemecka(61) estimated the population of Azotobacter in the order of 8,000 per gram of soil from plots that permanently received non-nitrogenous artificial fertilizer. The count was much lower when nitrogenous fertilizer was added. Brown, Burlingham and Jackson(10) found that the natural population of Azotobacter is from 20 to 8,000 per gram dry weight of soil.

Clostridia are more abundant in soil than Azotobacters. Meiklejohn(42) reported numbers of 10^3 to 10^5 per gram, and Abdel-Malek and Ishac(1) reported 10^8 per gram of dry soil. Little is known about the organic compounds specially favorable for the growth of Clostridia in soil, but they have an ability to utilize a wide range of carbohydrates and sugars, such as pentoses, hexoses, di- and polysaccharides and pectin substances(34). During the metabolism of organic compounds by Clostridia, lactic acid, acetic acid and many other organic acids are produced, most of which can be utilized by Azotobacters. Thus Clostridium spp. and Azotobacter spp. can enter in good relation with each other. It was

stated by Jensen(34) that a certain degree of anaerobiosis is necessary for active nitrogen fixation in soil. Partial anaerobiosis is probably common in soils since Azotobacters and other aerobes and facultative microorganisms can lower the oxygen partial pressure of soil. This permits anaerobic organisms such as Clostridia to initiate growth and fix nitrogen. Clostridia are of great importance in nitrogen fixation since they are more numerous than other nitrogen fixers in most soils and have world-wide distribution.

Biochemistry of non-symbiotic nitrogen fixation.

Nitrogen fixation is a reductive and energy consuming process. The key compound in supplying energy and reducing power in anaerobic Clostridia is pyruvic acid, which undergoes a phosphoroclastic breakdown into acetylphosphate (an energy-rich product), carbon dioxide and hydrogen gas:

$$CH_3.CO.COOH + H_3PO_4 \longrightarrow CH_3.CO.O.H_2PO_3 + CO_2 + H_2 \quad (43)$$

In this phosphoroclastic reaction, Ferredoxin(Fd), an electron transfer protein, co-factors thiamine pyrophosphate (TPP) and coenzyme A (CoA) are required (43,44). In nature, pyruvic acid can be synthesized from exogenous carbohydrate via respiration. The schematic diagram for the process of nitrogen fixation is shown in Fig. 1 (30,44). The first stable product of fixation is ammonia. This has been confirmed by Carnahan and his co-workers(17) who found that $^{15}N_2$ taken up by Clostridium pasteurianum cell free extracts could be recovered quantitatively as $^{15}NH_3$. In the presence of ammonia in bacterial culture, nitrogen fixing activity is inhibited due to the preferential assimilation of ammonia-nitrogen(46).

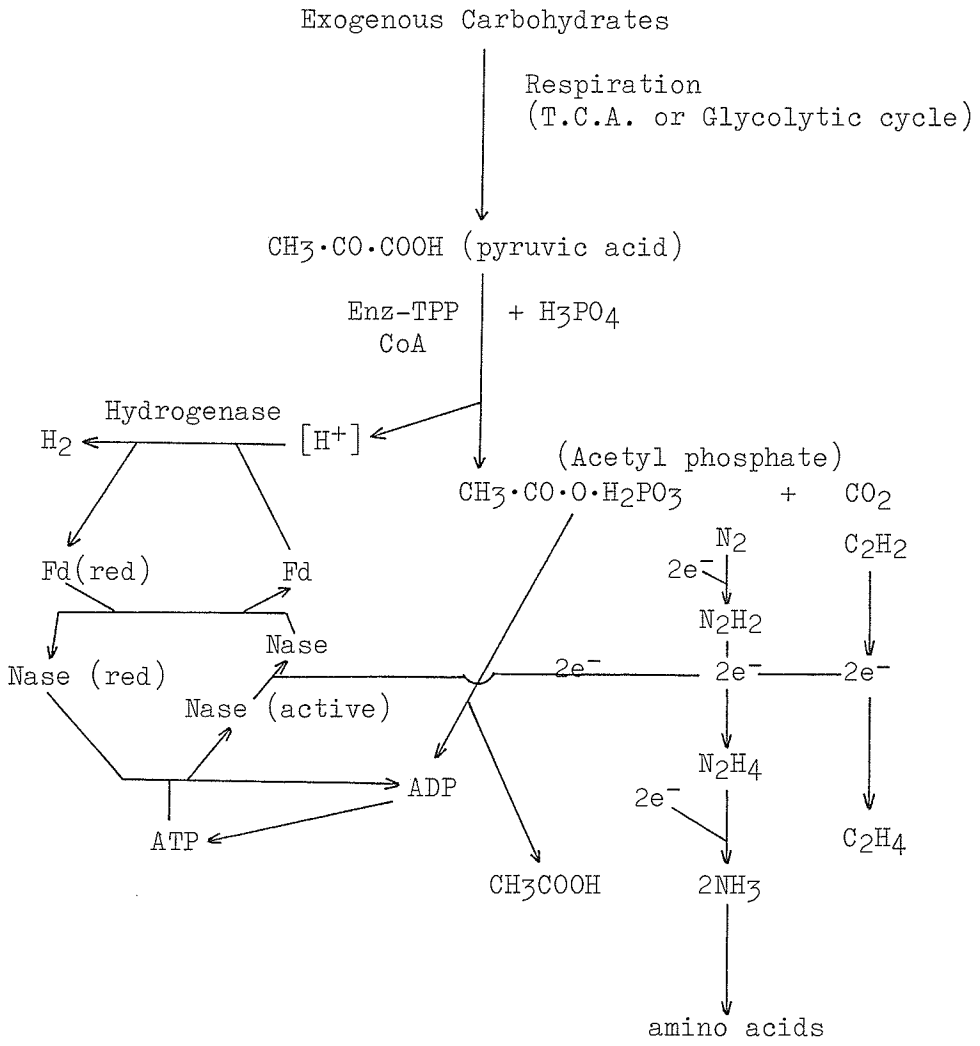


FIG. 1. SCHEMATIC DIAGRAM OF THE PROCESS OF NITROGEN-FIXATION.

Two important enzymes, nitrogenase and hydrogenase, are involved in nitrogen fixation. Hydrogenase reversibly catalyses the conversion of hydrogen ions to molecular hydrogen, and nitrogenase catalyses the reduction of molecular nitrogen. These enzymes have been detected in most of the nitrogen fixers. Nitrogenase isolated from C. pasteurianum and A. vinelandii is a cold labile, oxygen sensitive, acidic complex made up of two protein fractions, neither of which alone can reduce nitrogen(11,12,25). One protein contains non-heme iron and molydenum and the other fraction contains only non-heme iron(12,45). Fig. 1 shows that nitrogenase catalyses stepwise by two electron reductions, and a total of six electrons are required to reduce a nitrogen molecule to two molecules of ammonia. The nitrogenase system of Azotobacter is not much different from that of Clostridia since a crude Clostridium hydrogenase system will support fixation by Azotobacter nitrogenase(11).

Methods of determining nitrogen fixation.

A. Total nitrogen analysis.

In early studies on nitrogen fixation, determination of total nitrogen by Kjeldahl method was used. The results obtained by this method were frequently inconclusive, due to the insensitivity of the method and because it does not determine all the nitrogen present in some materials. Burris and Miller(14) stated that inaccuracies of the Kjeldahl method have frequently suggested fixation of nitrogen by germinating seeds, Rhizobium independent of its host, non-leguminous plants and other biological agents whose ability to fix nitrogen is questionable(14).

B. ^{15}N tracer technique.

The limitations imposed by the Kjeldahl method in the studies of

nitrogen fixation were remedied by the application of the stable nitrogen isotope, ^{15}N, as a tracer by Burris and Miller in 1941(14). Results obtained by this method are quantitative and reproducible. This method has been used to study the N-fixing capacity of soil(19,21) and of bacterial cell-free extract(9,16). Although this method is sensitive, a few drawbacks exist. The drawbacks are that $^{15}N_2$ gas is very expensive and mass spectrometer which is used to analyze the gas samples is expensive and not easily operated, and also the whole process is very time consuming.

C. ^{13}N radioisotope incorporation technique.

Radioisotope ^{13}N was introduced by Nicholas and his co-workers(47) in 1961 for the study of nitrogen fixation. A quantitative study of N-fixation by this method is rather difficult since the specific activity of the nitrogen sample activated by a cyclotron is very difficult to determine. Therefore this method is a qualitative determination of N-fixation. The usefulness of this technique is also limited by the short half life of ^{13}N of approximately 10 minutes. As a result, not only must fixation experiments be of short duration but a cyclotron must be available for preparation of the isotope.

This method has been used by Campbell and his co-workers(15) as a rapid screening technique for establishing the nitrogen fixing potential of 150 isolates of subarctic soils.

D. Acetylene reduction method.

This is an indirect method of determining N-fixation. In 1966 Schollhorn and Burris(56), and Dilworth(23) independently observed the inhibitory effect of acetylene on nitrogen fixation with production of

ethylene (C_2H_4) in cell free extract of C. pasteurianum. Later acetylene reduction was also observed with A. vinelandii. The inhibitory effect was reported to be reversible(23), which means that nitrogen fixation continues in absence of C_2H_2, and competitive(56). The requirements for reduction are the same as those for nitrogen fixation, namely, ferredoxin for electron transfer, adenosine triphosphate (ATP) as energy source and the reduction enzyme, nitrogenase(23). In 1967, Hardy and Knight(28) proposed the application of C_2H_2 reduction to assay the nitrogen fixing activity. The ethylene produced by reduction of acetylene may be detected by gas chromatograph. Since the proposal by Hardy and Knight, the acetylene reduction method has been carried out by many workers and confirmed to be a good index of N-fixation. The usefulness of this method is supported by Koch et al.(39,40) with cell free extracts of soybean root nodules; in situ studies on lake water and soil by Stewart et al.(59); and nitrogen fixation by Mycobacterium flavum 301 by Biggins and Postgate(9). Procedures for this method have been suggested by Schollhorn and Burris(57), and Hardy and his co-workers(29).

Acetylene reduction occurs because acetylene has the closest analogous structure to nitrogen, although they have different chemical properties. Acetylene is isoelectronic and isosteric with nitrogen and thus it should fit easily into the active site of nitrogen fixing enzyme, nitrogenase. Therefore this method is sometimes referred to as the assay for the nitrogenase activity. During the reduction of 1 molecule of N_2 to $2NH_3$ six electrons are required while acetylene requires two electrons for the reduction(59)(Fig.1). After a series of experiments using C_2H_2 reduction method, Hardy and his co-workers concluded that the C_2H_2-C_2H_4 assay of

nitrogen fixation is sensitive, universal, specific, rapid, simple, economical and quantitative(29).

Nitrogen fixation capacity of soil as determined by ^{15}N tracer method.

Delwiche and Wijler(21) suggested that molecular nitrogen was incorporated in certain field soils under aerobic condition providing that glucose was added, and they found that 1 gm glucose added to 100 gm of soil (1% glucose) was required for fixation of approximately 40 pounds per acre in 40 days at 21°C. Recently, Chang and Knowles(19) concluded from their experimental data that fixation was more pronounced under anaerobic conditions, whether glucose was added or not, and fixation also occurred under aerobic conditions in significant amount only if glucose was supplied. The samples of Chang and Knowles were supplemented with 1% glucose and incubated at 30°C for 29 days. It was later suggested by Knowles(38) that much of the biological nitrogen gain occurring in natural and cultivated soils might be associated with periods or regions of anaerobiosis or with localized available energy sources.

MATERIALS AND METHODS

Preparation of soil samples

A soil sample was collected from the Ap horizon of an Orthic Black soil in a cultivated field near Portage la Prairie, Manitoba, during the month of September, 1968. Immediately after collection, the sample was air dried on polyethylene sheets, ground to pass a 2 mm sieve and then stored in polyethylene bags at 4°C. Characteristics of the soil are outlined below:

Texture	: clay loam
pH (1:1 soil water extract)	: 7.5
Conductivity of 1:1 soil water extract	: 0.25 mmhos
NO_3-N	: 1.6 ppm
$NaHCO_3$ extractable P	: 6.2 ppm
Exchangeable K	: 270 ppm
Field capacity (1/3 atmosphere suction)(%H_2O)	: 27%
Total N as determined by Kjeldahl digestion	: 0.33%

All analyses, except field capacity and total nitrogen, were conducted by the Provincial Soil Testing Laboratory.

To study the effect of glucose on nitrogen fixation, 1 ml of solutions containing 0.4%, 2%, 10% and 50% glucose and 1.7 ml of distilled water to bring the moisture content to field capacity were added to four 10 gram soil samples providing soil samples with glucose concentrations, on soil weight basis, of 0.04%, 0.2%, 1% and 5%, respectively. The samples so prepared were kept in a refrigerator at 4°C for 2-3 hours to establish uniform distribution of glucose throughout the soil samples.

Soil pH determination.

Two grams of moist soil was mixed with 20 ml of water and stirred vigorously for 15 minutes. The pH was measured by Coleman Metrium III pH meter. This is the method described by Jackson(32) except that the

stirring time was reduced from 30 minutes to 15 minutes.

Incubation for ^{15}N-fixation.

The apparatus used for incubation of the soil is illustrated in Fig. 2. This apparatus was so designed that when placed in the reciprocating shaker described below and moved through an arc of 60 degrees, the gas was forced back and forth through the soil in B as the KOH solution flowed from one end to the other of the tube A. Thus the soil was continuously aerated with the gas mixture and carbon dioxide produced during incubation was absorbed by the KOH. The 1% KOH solution also maintained a high humidity in the gas mixture so that moisture content of the soil remained nearly constant during incubation.

The volume of the incubation vessel was calculated from the weight of water it held. The vessels varied slightly in size but ranged from 90 to 95 ml. After addition of 10 grams of soil (particle density of 2.5) and 35 ml to 40 ml of 1% KOH, there was approximately 50 ml available for the gas mixture used.

Following procedure was used for the introduction of the desired gas mixture for the incubation purpose:

1) The incubation vessel was well evacuated through X, flushed twice with argon, and then evacuated to a pressure lower than 10^{-3} Torr.

2) Oxygen was introduced to give a partial pressure of 0.2 atmosphere as indicated by a manometer attached to the vacuum line.

3) The pressure was brought up to 1 atmosphere with argon, and clamp G was tightened to close the system.

4) The partial pressure of the incubation vessel was reduced to approximately 0.8 atmosphere by removing 10 ml of the gas mixture with 1/2 inch hypodermic needle fitted onto a 30 ml hypodermic syringe (B-D Gale).

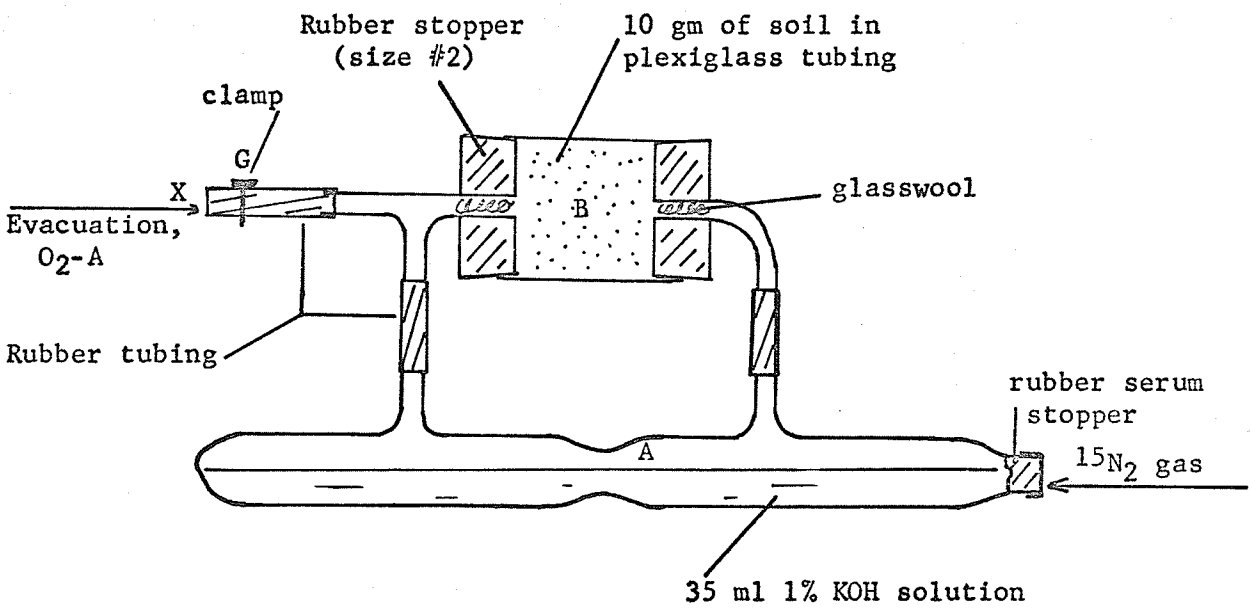
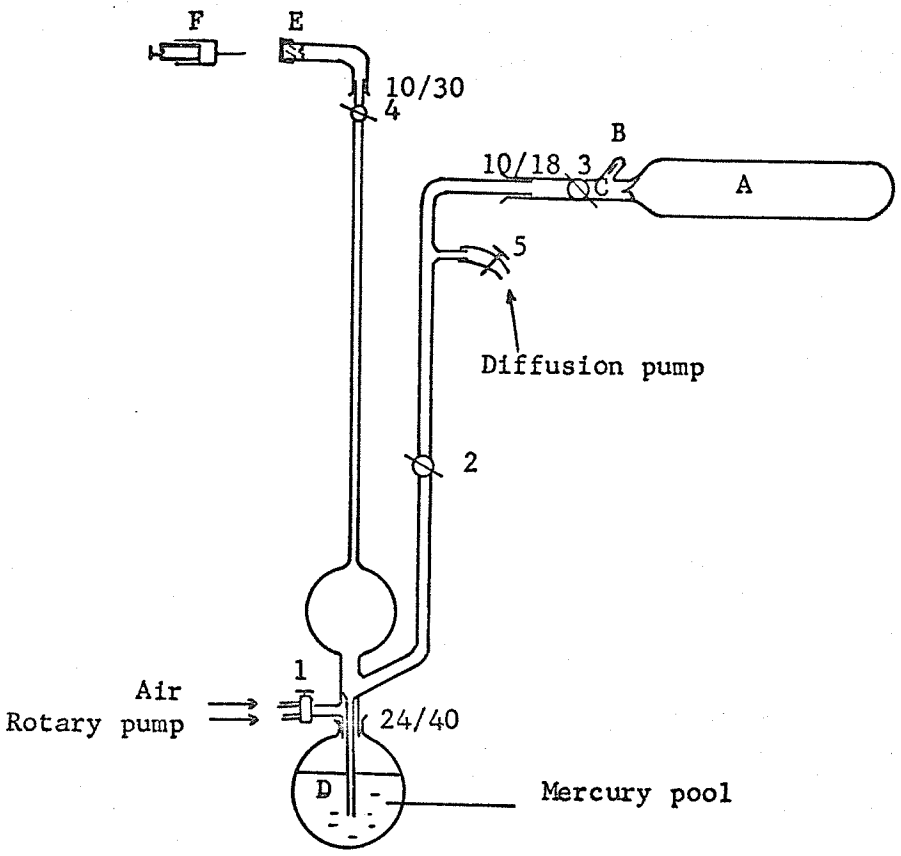


FIG. 2 - INCUBATION VESSEL FOR DETERMINATION OF N-FIXATION BY ^{15}N TRACER TECHNIQUE.

5) 10 ml of enriched $^{15}N_2$ gas was immediately transferred from the $^{15}N_2$ gas stock container into the vessel.

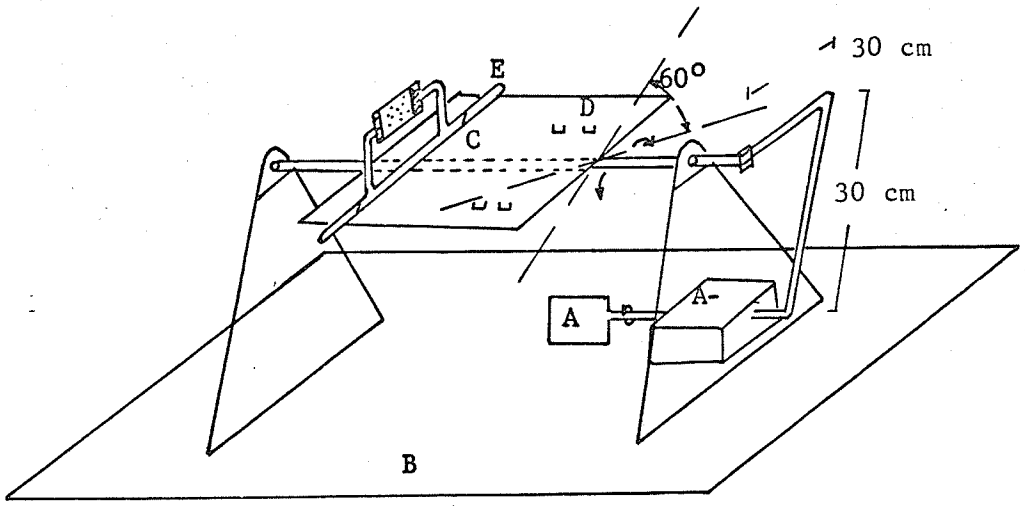
The technique of transferring 10 ml $^{15}N_2$ gas from the stock container into hypodermic syringe was carried out with the aid of modified Toepler pump (Fig. 3) similar to that of Cho and Haunold(20). The modified Toepler pump was evacuated by the rotary pump for 5 minutes with all stopcocks opened. Evacuation was completed using the double stage mercury diffusion pump. This took 30 minutes. Stopcocks #1, #3, and #5 were closed and the glass seal of the stock nitrogen-15 gas sample container was broken by the metal strip with the aid of a magnet while the Toepler pump was under vacuum. Stopcock #3 was opened so as to let enough $^{15}N_2$ gas flow from the stock container to fill the system. This was indicated by the vigorous bubbling of mercury in the mercury pool at which stopcock #3 was closed. Stopcock #1 was then opened to the air inlet, and air flowed into the system to balance the $^{15}N_2$ gas pressure. This was indicated by the rise of mercury level through the capillary. A hypodermic syringe supported by a ring was then inserted through the rubber serum stopper E. Pressure was applied with a hand pump via air inlet causing mercury to rise in the capillary and increasing the pressure of $^{15}N_2$ gas and gradually pushing out the plunger of the syringe at E, thereby filling it with nitrogen-15 gas. When gas had filled the syringe, it was removed and gas was transferred to the incubation vessel via its rubber serum stopper.

The final concentration in the artificial atmosphere was 0.64 atm A, 0.16 atm O_2, and 0.2 atm $^{15}N_2$ gas. The vessel was then placed on a reciprocator, illustrated in Fig. 4, for 14 days incubation at either



A = $^{15}N_2$ gas stock container
B = Metal strip
C = Glass seal
D = Mercury pool
E = Rubber serum stopper
F = 30 ml hypodermic syringe (B-D Yale)
⊗ = High vacuum stopcocks

FIG. 3 - MODIFIED TOEPLER PUMP FOR ^{15}N GAS TRANSFER.



A- = Speed reducer with ratio 900:1.
A = Electric Motor (1800 r.p.m.)
B = Board (164 x 60 x 1½ cm^3)
C = Board for holding incubation vessels (120 x 30 x 1 cm^3)
D = Clamps
E = Incubation vessel.

FIG. 4 - THE RECIPROCATOR.

15°C, 25°C, or 35°C. A speed reducer having a ratio 900:1, and an electric motor having 1800 r.p.m. were required for the operation of the reciprocator. The two 30 cm metal bars were joined in such a way that the board C welded on the horizontal metal bar was free to move in an arc of 60 degrees. Small clamps were affixed on the board to hold the incubation vessels in a vertical position.

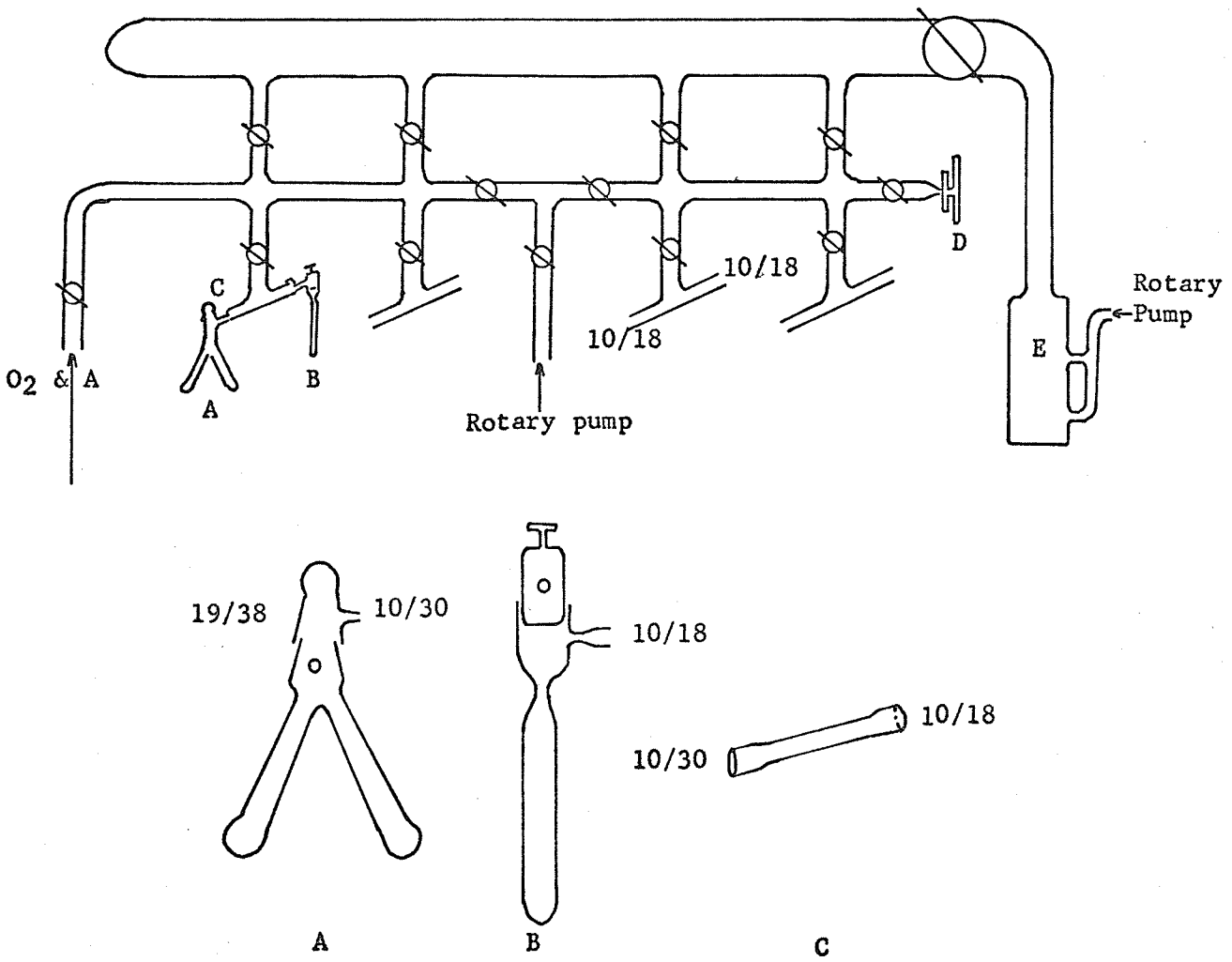
Kjeldahl determination of nitrogen.

The procedure followed was an adaptation of that described by Bremner(16). This is the Kjeldahl method modified to include the determination of NO_3^- and NO_2^-. A 5-gram moist sample of soil was transferred into a Kjeldahl flask. The water content was determined on a separate sub-sample. Thirty ml of 5% potassium permaganate solution, 30 ml of 50% sulfuric acid, 2 drops of octanol, 0.5 g finely ground iron powder, and boiling chips were added to the flask. The solution was boiled for 15-20 minutes until initial effervescence stopped. The solution was cooled, 20 ml conc. H_2SO_4 and a package of Kel-pak (contains 0.3 g $CuSO_4$ and 10.0 g K_2SO_4) were added. The sample was heated again for 2-3 hours until the color of solution became yellowish-green. After 2-3 minute cooling, 300 ml distilled water was added and the solution was allowed to cool to room temperature. Then 60 ml conc. NaOH was added slowly. The ammonia released was distilled into 25 ml of 0.1-N H_2SO_4 containing methyl red indicator. Approximately 150 ml of distillate was collected. Total nitrogen was determined by back titration with standard NaOH. Following titration the solution was made acid with 1 drop of conc. H_2SO_4, and reduced to a volume of approximately 10 ml by evaporation, and stored in a refrigerator. The final step consisted of conversion of the ammonium ion to nitrogen gas as described below.

Preparation of nitrogen gas from ammonia for mass spectrometric analysis.

In order to achieve a minimum contamination of sample nitrogen with atmospheric nitrogen, the conversion of ammonia to nitrogen gas was carried out in a vacuum system. Fig. 5 shows the vacuum line together with the Rittenberg reaction vessel and gas sample container in their proper position. Air in the vacuum line was first evacuated with the aid of the rotary pump, and then evacuated to a pressure lower than 10^{-3} Torr via the double stage mercury diffusion pump. The pressure was monitored with a micro-McLeod gauge. The Rittenberg reaction vessel was free to rotate 360° horizontally, and the Rittenberg vessel cap also could be turned 360° vertically while it was on the vacuum line.

One arm of the reaction vessel was filled with 3.0 ml of the condensate obtained from Kjeldahl digestion, and the other arm was filled with NaOBr-KI solution (method of preparation was same as by Bremner(7). In order to attain high vacuum without evaporational loss of liquid, the Rittenberg vessel was frozen with liquid nitrogen during evacuation of atmospheric gas mixture. After the solutions were defrosted, they were mixed by tilting the vessel to a 60° angle. Evolution of nitrogen gas was noted by the vigorous bubbling of the solution during mixing. After the reaction subsided, the volume of the solution in both arms was equalized by transferring a portion into the empty arm. The Rittenberg vessel was frozen again in order to decrease the vapor pressure of water, and subsequently the formed nitrogen gas was transferred to a sample container with the aid of liquid N_2. After nitrogen gas was transferred, the gas sample container was removed from the vacuum line for mass spectrometric determination of ^{28}N-^{29}N abundance.



A = Rittenberg reaction vessel
B = Gas sample container
C = 10/30-10/18 adaptor
D = Virtis micro-McLeod gauge
E = Two stage mercury diffusion pump
⊗ = High vacuum stopcocks

FIG. 5 - SCHEMATIC DIAGRAM OF THE VACUUM LINE AND REACTION VESSELS.

The abundance of ^{15}N was expressed as atom percent ^{15}N which was calculated from equation:

$$\text{Atom percent } ^{15}N = \frac{100}{2R + 1}$$

in which $R = {}^{28}N/{}^{29}N$, and ^{28}N and ^{29}N were referred to the peak heights of mass to charge ratio (m/e) of 28 and 29, respectively. These peaks were detected by mass spectrometer. This equation holds true only if the equilibrium constant for the following reaction is 4: (7)

$$^{14}N^{14}N + {}^{15}N^{15}N \rightleftharpoons 2\ {}^{14}N^{15}N$$

$$\text{Keq.} = \frac{(^{14}N^{15}N)^2}{(^{14}N^{14}N)(^{15}N^{15}N)}, \quad \text{or} \quad \frac{(^{29}N)^2}{(^{28}N)\ (^{30}N)}$$

This value was proved to be true experimentally with $^{15}NH_4{}^{15}NO_3$ of known atom percent ^{15}N.

Contamination of gas samples.

1) Atmospheric nitrogen.

A sample of gas from an incubation vessel was transferred to gas sample container at 0 time and after 48 hours and 96 hours for mass spectrometric determination of ^{28}N, ^{29}N, and ^{30}N.

2) Adsorbed nitrogen in glassware and mass spectrometer.

Unlabelled NH_4NO_3 and 1.5 atom percent $^{15}NH_4NO_3$ solutions were used as test reagents. Rittenberg reaction vessels numbered 1, 2, and 5 were filled unlabelled ammonium solution and vessels numbered 3, 4, and 6 were filled with ^{15}N labelled ammonium solution. The vessels were paired by gas sample containers having the same numbers. After nitrogen gas was collected, gas samples were analyzed by mass spectrometer in the order 1, 2, 3, 4, 5, and 6, respectively. Subsequently peak heights were measured and atom percent ^{15}N were calculated.

Incubation for acetylene reduction.

The incubation vessel (Fig. 6) designed was slightly different from that of ^{15}N-fixation incubation vessel. Rubber connections were avoided because acetylene and ethylene might diffuse through the rubber tubing. The method of evacuation and the O_2-A introduction were the same as ^{15}N fixation except that these were done with a thick needle inserted through the large rubber stopper A. Ten ml of C_2H_2 was transferred directly from the main acetylene gas cylinder with a hypodermic syringe to the vessel through the rubber serum stopper E. The samples so prepared were incubated for 8 days, or 14 days, with daily analysis of gas mixture.

Detection and estimation of ethylene.

A volume of 50 µl gas mixture was taken from the incubation vessel and introduced into the gas chromatograph via the rubber septum of the heated injector tube with a 50 µl Hamilton gas-tight microsyringe. Gas mixture was then mixed with carrier gas, argon, in the gas chromatograph. As the carrier gas pushed the sample into the Porapak T chromatographic column, it partitioned directly from the gas phase into the solid amorphous polymer. Porapak T column contained only the porous polymer beads having a mesh size of 50-80 microns. The rate at which the sample travelled through the column was determined by the carrier gas flow rate. Each gas in the sample travelled at a characteristic rate, and the gases were completely separated by the time they reached the detector. The gas chromatograph (Varian Aerograph 1200 series) used here was equipped with H_2-flame ionization detector which indicated the presence and concentration of each gas in the gas mixture by showing peaks on the recorder chart

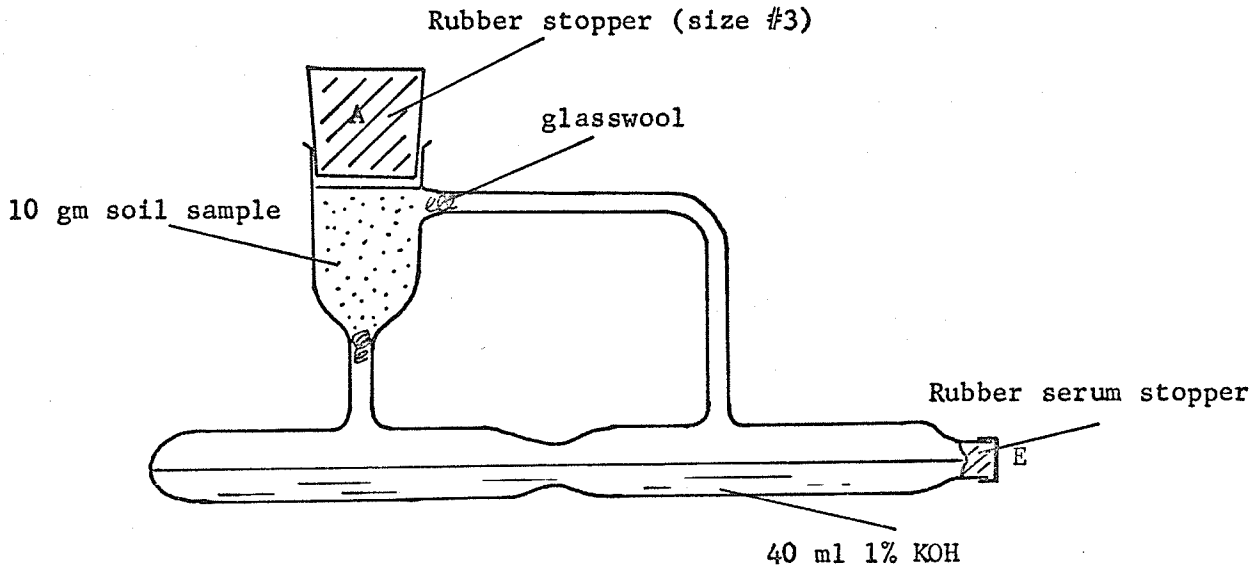


FIG. 6 - INCUBATION VESSEL FOR C_2H_2-C_2H_4 ASSAY.

(Varian Aerograph Model 20 strip chart recorder). The type of gas was identified by its retention time or the length of time a gas remained in the column. The elution time for C_2H_4 and C_2H_2 under the standard conditions listed below was 45 seconds and 1 minute 45 seconds, respectively. The peaks recorded were approximately symmetrical and the peak heights were measured to represent the concentration of the gases.

A standard curve for quantitative estimation of C_2H_4 was established using pure C_2H_4 gas. This standard curve is accurate and reliable only if certain standard conditions are established and maintained throughout the entire study. The standard conditions were as follows:

Detector	:	H_2-flame ionization
Column material	:	Acon copper tubing (6 ft. × 1/8 in.O.D.)
Column packing material	:	Porapak T (Water Association)
Injector temperature	:	120°C
Detector temperature	:	160°C
Oven temperature	:	50°C
Argon flow rate	:	27 ml/min.
Hydrogen flow rate	:	21 ml/min.
Oxygen flow rate	:	103 ml/min.
Recorder chart speed	:	30 in/hr.

RESULTS AND DISCUSSIONS

The feasibility of using $\frac{100}{2R + 1}$ for calculation of ^{15}N abundance.

Equilibrium constant and atom percent ^{15}N of the samples were calculated, and are shown in Table 1. These values indicated that the probability of formation of respective N_2 molecules from NH_3 due to oxidation was in accordance with statistical law, and atom percent ^{15}N could be calculated by the equation above.

There might be some argument whether a double collector system or a single collector system should be used to measure the abundance of ^{15}N. A double collector system permits simultaneous collection of ions of different masses on two separate collectors, while in a single collector system, the ions of different masses were focused to fall on a single collector by varying the acceleration voltage. Data shown in Fig. 7 suggest that the single collector system is more accurate. Data with the double collector were consistently lower than those obtained by the single collector system. This may have been due to over-estimation of mass 28 by the double collector system. Fig. 7 shows that the higher the atom percent ^{15}N, the greater is the difference between the methods. The atom percent ^{15}N value determined by single collector system is exactly the same as that of the expected value. Therefore gas samples obtained in the undergoing studies were determined by mass spectrometer equipped with a single collector.

Contamination of gas samples.

1) Atmospheric nitrogen.

The results shown in Table 2 indicated that the atom percent ^{15}N

TABLE 1. EQUILIBRIUM CONSTANT OF A KNOWN SAMPLE.

$^{15}NH_4\ ^{15}NO_3$	Peak Heights			*K	Atom % ^{15}N	
	^{28}N	^{29}N	^{30}N		**Calculated	Expected
2 mg	52	6.1	0.178	4.02	5.54	5.50
5 mg	42.3	4.95	0.145	3.99	5.53	5.50

* K (equilibrium constant) = $\frac{[^{29}N]^2}{[^{28}N]\ [^{30}N]}$

** atom % N^{15} = $\frac{100}{2R + 1}$ where $R = \frac{[^{28}N]}{[^{29}N]}$

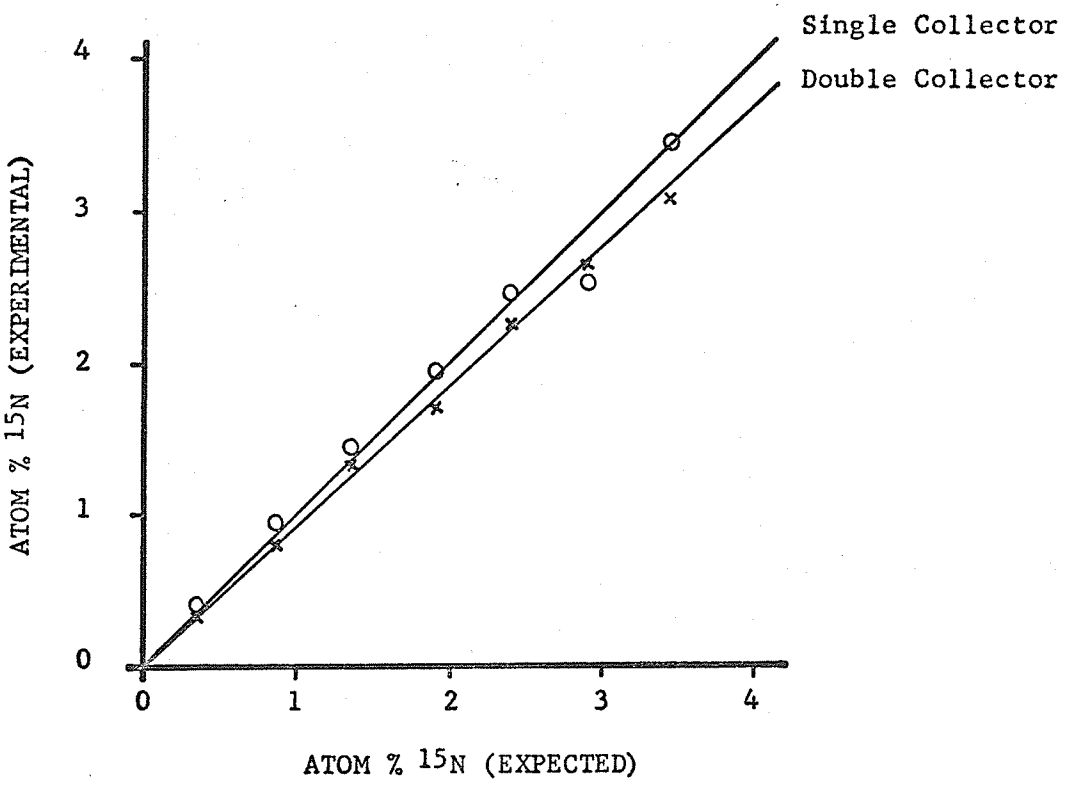


FIG. 7 - ATOM % ^{15}N AS DETERMINED BY DOUBLE COLLECTOR AND SINGLE COLLECTOR.

TABLE 2. ATOM % ^{15}N IN INCUBATION VESSEL AFTER 48 AND 96 HOURS INCUBATION

Trials	Atom % ^{15}N*				Expected
	Time (hours)				
	0	48	0	96	
1	52.5	52.8	53.2	51.6	53.2%
2	52.4	53.0	53.0	52.8	

* atom % $^{15}N = \frac{2[^{30}N] + [^{29}N]}{2[^{28}N + ^{29}N + ^{30}N]}$

remained approximately constant after 48 hours and 96 hours incubation. This suggested that the method of $^{15}N_2$ gas transfer was accurate and that contamination by atmospheric nitrogen did not occur.

2) Adsorbed nitrogen in glassware and mass spectrometer.

The result in Table 3 shows that the experimental value of atom percent ^{15}N is exactly the same as that of the expected value. This indicated that there was no memory effect from the glassware or the mass spectrometer, and the results obtained in the studies should be accurate and reliable.

Natural abundance of ^{15}N in Portage clay loam.

Natural abundance of ^{15}N in atmospheric nitrogen is 0.366 atom percent. This value might be different in soil. Bremner and his co-workers(18) determined nitrogen-15 content on a wide variety of soil samples, and concluded that the atom percent ^{15}N of soil nitrogen is usually higher than that of atmospheric nitrogen, but it rarely exceeds 0.380. This prompted the determination of the abundance of ^{15}N for the Portage clay loam. The mean atom percent ^{15}N on the basis of 16 replicates was determined to be 0.367 with a standard deviation of $\pm$ 0.006. (Table 4).

Effect of partial pressure of Nitrogen upon N-fixation.

Nitrogen fixed in 14 days at various partial pressures of nitrogen at 25°C with 1% glucose is shown in Fig. 8. The N-fixation increased gradually with increasing partial pressure of nitrogen from 0.1 atm. to 0.25 atm. and then decreased. At 0.4 atm., the amount of nitrogen fixed was only one third of that with 0.1 atm. N_2. The observation here is contrary to that suggested by Koch and Evans(39) working with soybean root nodules.

TABLE 3. EXPERIMENTAL TEST OF MEMORY EFFECT IN GLASSWARE AND MASS SPECTROMETER BY ALTERNATING NATURAL AND ENRICHED N_2 SAMPLES

Gas Sample Container	Atom % ^{15}N	
	Expected	Experimental
1	0.366	0.366
2	0.366	0.364
3	1.50	1.55
4	1.50	1.53
5	0.366	0.364
6	1.50	1.41

TABLE 4. MEAN VALUE DETERMINATION OF THE NATURAL ABUNDANCE OF ^{15}N IN PORTAGE CLAY LOAM AND THE STANDARD DEVIATION

Sample # (N)	atom % N^{15} (x)	x^2
1	0.3699	0.1368
2	0.3693	0.1363
3	0.3595	0.1292
4	0.3612	0.1305
5	0.3621	0.1311
6	0.3736	0.1396
7	0.3672	0.1348
8	0.3612	0.1305
9	0.3706	0.1373
10	0.3689	0.1361
11	0.3719	0.1383
12	0.3680	0.1354
13	0.3810	0.1452
14	0.3618	0.1309
15	0.3661	0.1340
16	0.3640	0.1324
$\sum$ 16	5.8763	2.1586

$(\sum x)^2 = 34.5309$

S = Standard deviation = $\dfrac{x^2 - (\sum x)^2/N}{N - 1}$

$= 0.00564$

Mean atom % $^{15}N = 0.367$

Significance of the values,

(a) for 99% = 2.58 S
= 0.0146

(b) for 95% = 1.96 S
= 0.0110

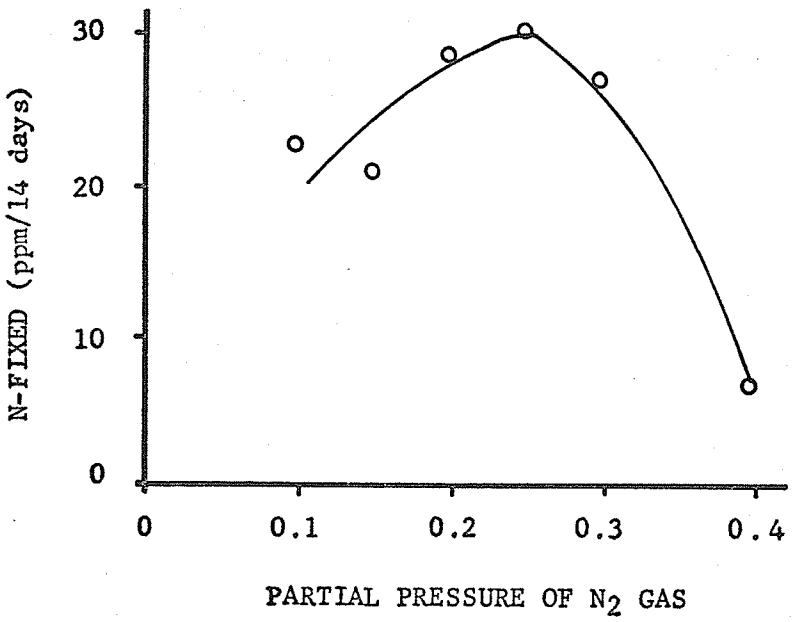


FIG. 8 - EFFECT OF N_2 PARTIAL PRESSURE ON N-FIXATION AT 25°C, 1% GLUCOSE

They reported that maximum fixation was obtained with 0.1 atm. N_2, and further addition did not increase the rate of fixation. For the purpose of subsequent investigations, the partial pressure of 0.2 atm. was chosen.

Effect of glucose concentration on nitrogen fixation at 15°C.

Atom percent ^{15}N of the incubated soil was converted to atom percent excess ^{15}N and the values greater than 0.015 atom present ^{15}N excess were taken as indicating significant nitrogen fixation. The nitrogen fixed was then calculated by the following equation:

$$\text{Amount of N fixed} = \frac{(\%\ \text{nitrogen in soil})(\text{atom}\ \%\ ^{15}\text{N excess})(10^4)}{(\text{atom}\ \%\ ^{15}\text{N excess of the gas})}$$

The excess ^{15}N and the calculated amount of nitrogen fixed for 14 days of incubation at 15°C is shown in Table 5. The amount of N-fixed during 14 days without the addition of substrate is very small (2 ppm). The addition of small quantities of glucose (0.04% and 0.2%) enhanced nitrogen fixation, although the magnitude of increase over the control sample is not large. Increasing the quantity of glucose to 1% greatly accelerated nitrogen fixation. Fixation then amounted to 34.3 ppm N in 14 days or equivalent to 140 pounds of nitrogen fixed per acre per month. Further increase in the addition of glucose to 5% drastically decreased N-fixation. This suggests addition of glucose stimulated an increase in microbial activity. As the microorganisms multiplied, nitrogen was required for the synthesis of cell proteins. Nitrogen gas might have been preferentially metabolized by nitrogen fixers which were present in the soil sample. When glucose concentration is increased to 5%, there was little nitrogen fixed. A few reasons for this reduction in N-fixation at 5% glucose level might be suggested:

TABLE 5. THE EFFECT OF GLUCOSE ADDITION UPON NITROGEN FIXATION AT 15°C.*

% glucose	% Moisture		atom % ^{15}N excess	N-fixation ppm/14 days
	Initial	Final		
0	27.0	25.2	0.036	2.0
0.04	27.0	24.6	0.046	3.7
0.20	27.0	25.6	0.075	4.4
1	27.0	25.3	0.540	34.3
5	27.0	24.7	0.066	4.0

* atom % ^{15}N of the incubation gas = 53.2%

1) Osmotic effect.

It has been shown by Lipman and Sharp(41) that 0.5-0.6% sodium chloride and 1.25% sodium sulphate were toxic to nitrogen fixers, and Johnson and Guezi(36) showed that osmotic tension of salts reduced nitrate production. This osmotic effect might also apply to effect of nitrogen fixation in presence of high concentration of sugar. When glucose concentration was too high, there was a risk of dehydrating the bacterial cell and eventually death of bacteria occurred.

2) Bio-competition.

Addition of glucose would stimulate not only the growth of nitrogen fixers, but also the growth of the other non-nitrogen fixers such as actinomycetes and some fungi. Non-nitrogen fixers might overgrow the nitrogen fixers and render glucose unavailable to them.

3) Inhibition effect.

There might be some sort of inhibition effect on the enzyme systems that required for the complete reduction of nitrogen to ammonia and other amine acids of the cell material.

Effect of glucose concentration on nitrogen fixation at 25°C.

N-fixation at 25°C is shown in Table 6. The result at 25°C is quite different from that of 15°C. First, the amounts of N-fixed at lower levels of glucose addition and the check at 25°C are smaller than those at 15°C. Second, the amount of N-fixed at 5% glucose level at 25°C is greater than that at 1% glucose level. Under the condition investigated here, at low glucose concentration of 0.04% and 0.2%, the amount of nitrogen fixed is approximately the same as that of the check. This suggests that a small amount of glucose when present in soil may not be enough to stimulate vigorous microbial activity or this small

TABLE 6. THE EFFECT OF GLUCOSE UPON NITROGEN FIXATION AT 25°C.*

% glucose	% Moisture		atom % ^{15}N excess	N-fixation ppm/14 days
	Initial	Final		
0	27.0	24.3	0.015	0.94
0.04	27.0	25.3	0.010	0.65
0.2	27.0	23.8	0.027	0.62
1	27.0	24.7	0.451	28.7
5	27.0	25.2	0.560	33.5

* atom % ^{15}N of the incubation gas = 53.2%

amount when present might have been utilized by non-nitrogen fixers first and thus glucose was not available for growth of nitrogen fixers. The amount of nitrogen fixed in the presence of 1% glucose is approximately 29 ppm in 14 days. As glucose concentration increased from 1% to 5%, there is only a slight increase of fixation when compared with that of 1% sample. At 5% glucose concentration, the amount of nitrogen fixed is approximately 33.5 ppm in 14 days or 2.25 ppm per day. The result obtained here is comparable with that obtained by Bremner and Shaw(5) who observed a fixation of 1.5 ppm per day when soil sample (soil 5) was supplemented with 5% glucose. The findings here suggests that in order to obtain significant nitrogen fixation, 1% glucose or material equivalent to 1% glucose should be added.

The pH of soil sample during the course of incubation at 25°C was investigated. The result is shown in Fig. 9. There was an initial drop in pH from 7.5 to 6.5, and then pH increased gradually from 6.5 to approximately 7.1. Since CO_2 was being absorbed by 1% KOH, it seems probable that the drop in pH was due to the formation of organic acids, such as formic acid, acetic acid, and pyruvic acid. While these products were formed, other microorganisms began to metabolize the products but the rate of metabolism was slower than the rate of formation. At approximately pH 6.5, the rate of metabolism began to gain over the rate of acid formation, therefore pH of soil started to increase. As pH of soil gradually decreased from pH 7.5 to 6.5, there was a gradual increase of nitrogen fixed up to 29 ppm. Nitrogen fixing activity was detected during the first day of incubation and the 29 ppm N-fixed was almost reached in eight days (Fig. 9). This suggests there might be some very active nitrogen fixers in the sample, and the pH remained favorable for the growth

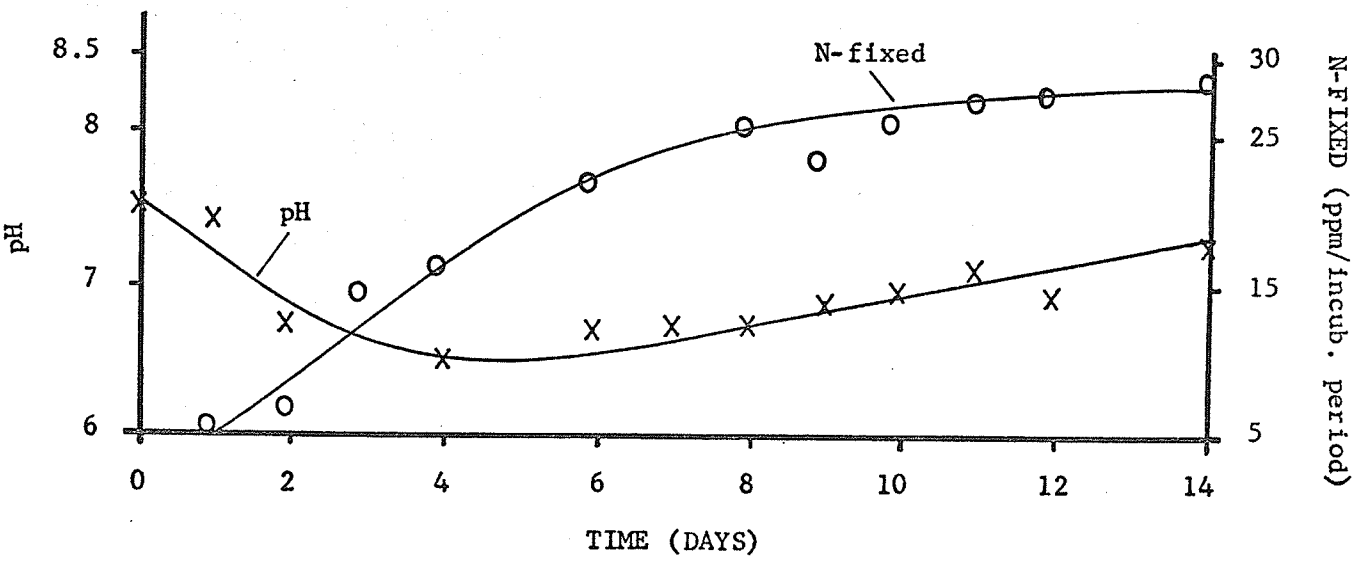


FIG. 9 - RATE OF N-FIXATION AND pH CHANGE AT 25°C, 1% GLUCOSE.

of nitrogen fixing organisms throughout the period of incubation.

The moisture content of the soil samples remained constant. This maintenance was effected by the presence of 1% KOH which effectively maintained the humidity of the closed system at approximately constant level.

Effect of glucose concentration at 35°C. on nitrogen fixation.

The result of the nitrogen fixation at 35°C is shown in Table 7. The general pattern of the effect of glucose addition upon N-fixation at this temperature is similar to that of 25°C except the magnitude of the fixation (Fig. 10). At the lower glucose levels and the check, the quantity of the N-fixed is similar to that at 25°C, but is smaller than that at 15°C. At higher glucose levels the fixation is lower than at 25°C. Fixation at 35°C exceeded that at 15°C with 5% glucose but the reverse was true with 1% glucose. The highest fixation at 35°C was 12.4 ppm when the sample was supplemented with 1% glucose.

The result suggests that 35°C is above the optimum temperature for N-fixation.

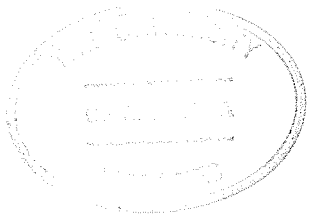
Standard curve of C_2H_4.

A regular method of constructing the standard curve by plotting the peak area against the concentration of gas mixture was not adopted in this study because it was found that the measurement of the area of an assymmetrical Gaussian Curve by the conventional method was not too accurate. Instead, it was empirically found that the peak height vs C_2H_4 concentration in log-log graph was reasonably satisfactory. The result of such a plot is shown in Fig. 11. At lower C_2H_4 concentration

TABLE 7. THE EFFECT OF GLUCOSE ADDITION UPON NITROGEN FIXATION AT 35°C.*

% glucose	% Moisture		atom % ^{15}N excess	N-fixation ppm/14 days
	Initial	Final		
0	27.0	22.9	0.021	1.28
0.04	27.0	24.2	0.021	1.32
0.2	27.0	25.3	0.025	1.56
1	27.0	25.1	0.198	12.4
5	27.0	23.8	0.197	11.3

* atom % ^{15}N of the incubation gas = 53.2%



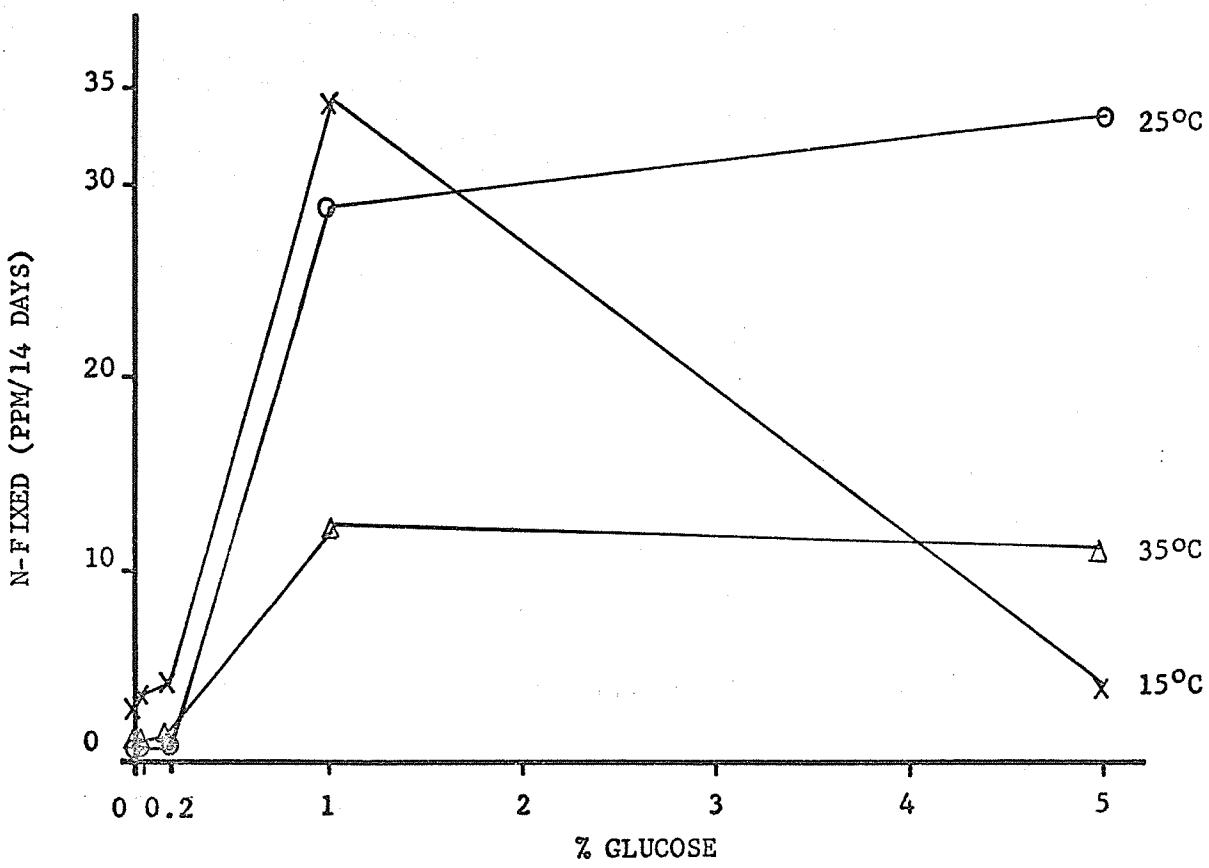
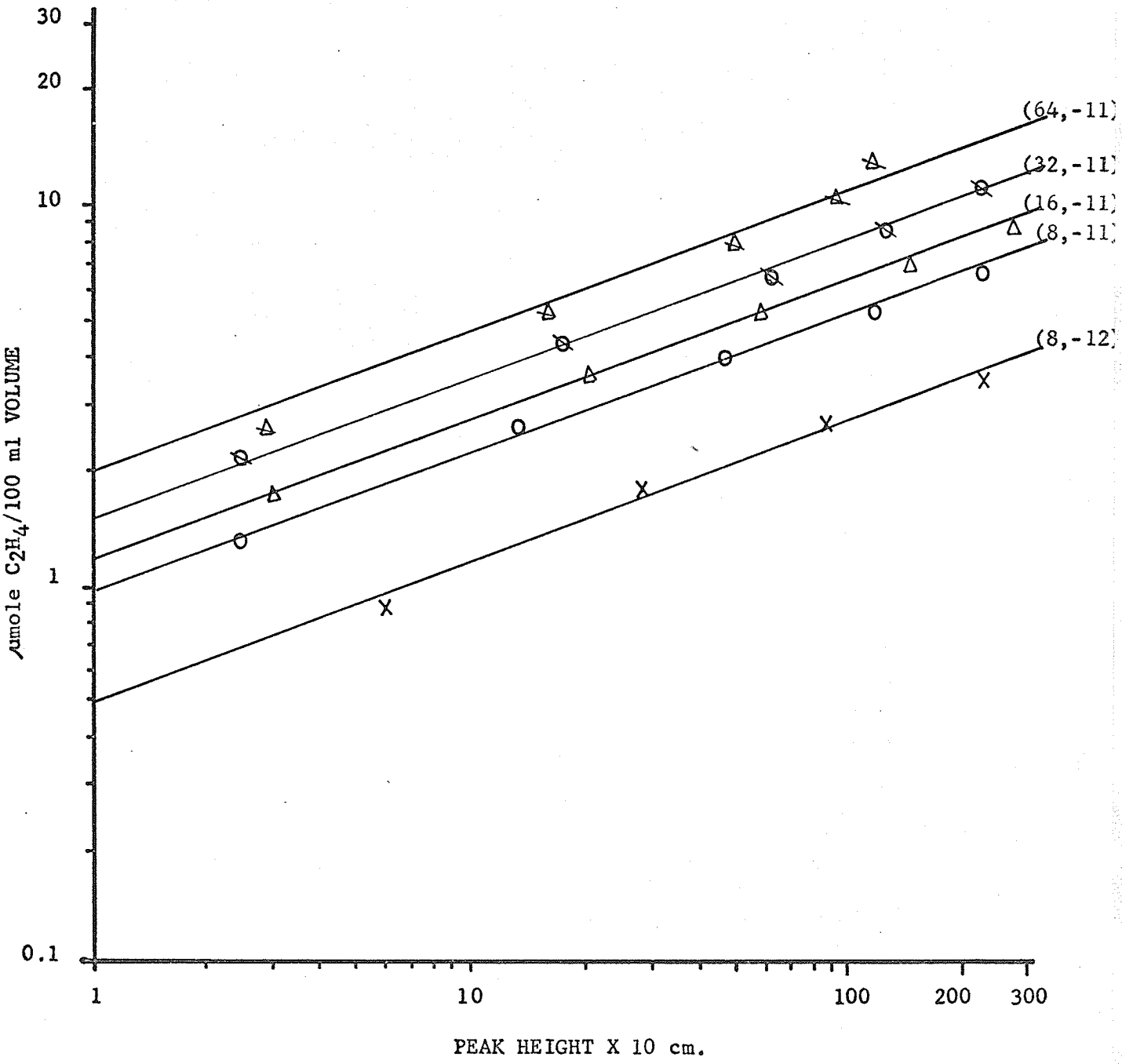


FIG. 10 EFFECT OF TEMP. & GLUCOSE ON N-FIXATION



* (X,Y) = (Attenuation, range - amp/Mv.)

FIG. 11 - STANDARD CURVES FOR QUANTITATIVE DETERMINATION OF C_2H_4.

the line is straight at (8,-12)* while at the high C_2H_4 concentration, the choice of (8, -11) was more satisfactory.

The effect of C_2H_2 concentration on production of C_2H_4.

A soil sample was incubated with 1% glucose for an 8-day period at 25°C in an incubating vessel containing 0.1 atm, 0.15 atm, 0.2 atm, 0.25 atm, 0.3 atm, and 0.4 atm. of C_2H_2.

The result indicates that there is a marked similarity between this curve (Fig. 12) and that of Fig. 8, the effect of partial pressure of nitrogen upon N-fixation, with the exception of a narrower optimum range for C_2H_2. The C_2H_4 production increased with increasing C_2H_2 until a partial pressure of 0.2 atm. was reached, and decreased thereafter. This is contrary to the results of Koch and Evans(39), who found the optimum C_2H_2 level for reduction by soybean root nodules to be 0.1 atm. of C_2H_2.

Both nitrogen fixation and C_2H_2 reduction seem to have decreased beyond 0.2 atm. for C_2H_2 but probably no decrease till 0.25 atm. with N_2. It may be due to inhibitory action of these gases upon the dehydrogenation reaction. It is not known in what mechanism these gases inhibit the reaction.

Rate of production of C_2H_4 at 15°C.

The cumulative quantity of C_2H_4 produced was converted to the quantity of nitrogen fixed by the following equation:

$$\left(\frac{X\ \mu mole}{3}\right)\left(\frac{28\ \mu g}{\mu mole}\right)\left(\frac{1\ g}{10\ g \times 10^6\ \mu g}\right) = 0.93X \text{ ppm, in which X is}$$

μmole of C_2H_4 formed. In a subsequent presentation, all the data

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* (X, -Y) refers to (attenuation, range in amp/mv)

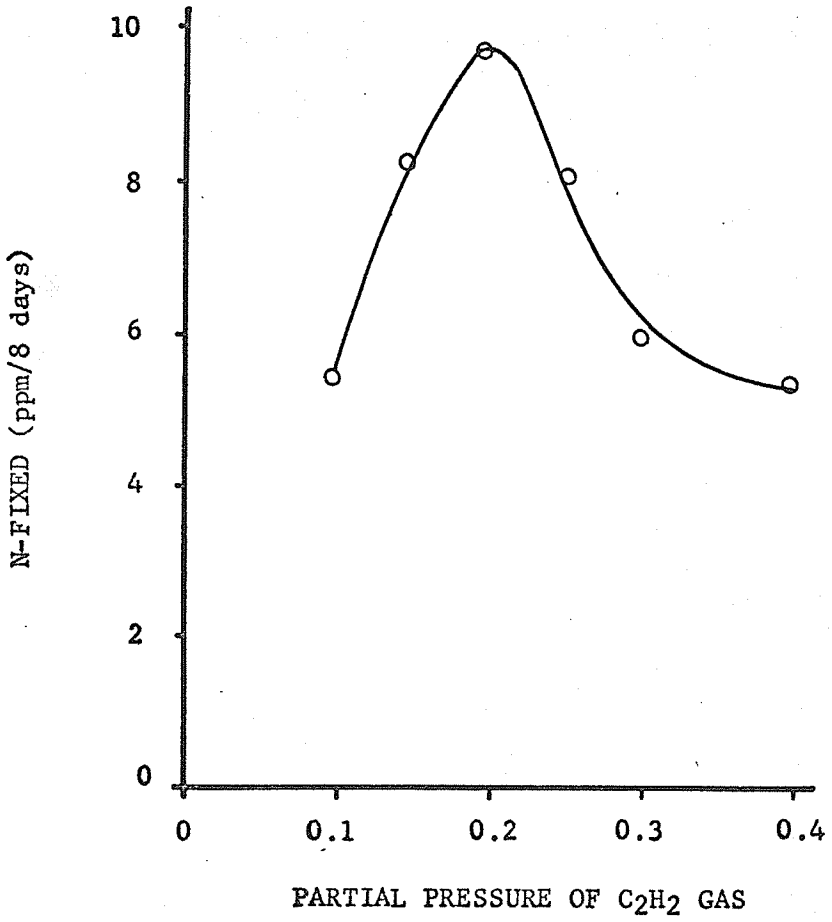

FIG. 12 - EFFECT OF C_2H_2 CONCENTRATION ON C_2H_4 PRODUCTION AT 25°C, 1% GLUCOSE.

concerning C_2H_4 produced will be presented in terms of N_2-fixed per million of soil calculated using above theoretical equation.

The amount of C_2H_4 produced during the 8-day incubation as affected by the addition of glucose was determined daily and the result obtained is listed in Fig. 13. It is evident that the addition of very small amounts, 0.04%, or very high amounts, 5%, did not result in significant production of C_2H_4 when compared with control sample. The quantity of C_2H_4 produced during the period of 8 days in these samples was less than 0.1 ppm. The samples supplemented with 1% glucose fixed approximately 11 ppm, and those supplemented with 0.2% glucose fixed 1.2 ppm during the same period.

Even with 0.2% and 1% glucose, it took 2 to 3 days for measurable C_2H_4 production to take place. The initial inactivity may be due to the adjustment of the microorganisms to new environment. The rate of C_2H_4 production from samples with 0.2% and 1% glucose after the initial inactive period is almost constant up to 8 days and corresponds to 0.15 ppm/day and 1.3 ppm/day respectively. This clearly shows that the 5-fold increase of glucose from 0.2% to 1% increased the rate of C_2H_4 production more than 5-fold, and the effect of glucose upon the C_2H_4 production is not directly related to the amount of glucose.

The decrease in fixation that occurred at 5% glucose was checked again using 14 samples incubated for periods ranging from 1 to 14 days, at 15°C. Daily determination of pH and the quantity of C_2H_4 produced was carried out. The result is shown in Fig. 14. The soil pH changes slightly from pH 7.5 to pH 7.35 at the first day of incubation and then remained constant through the period. The result is quite different from that obtained

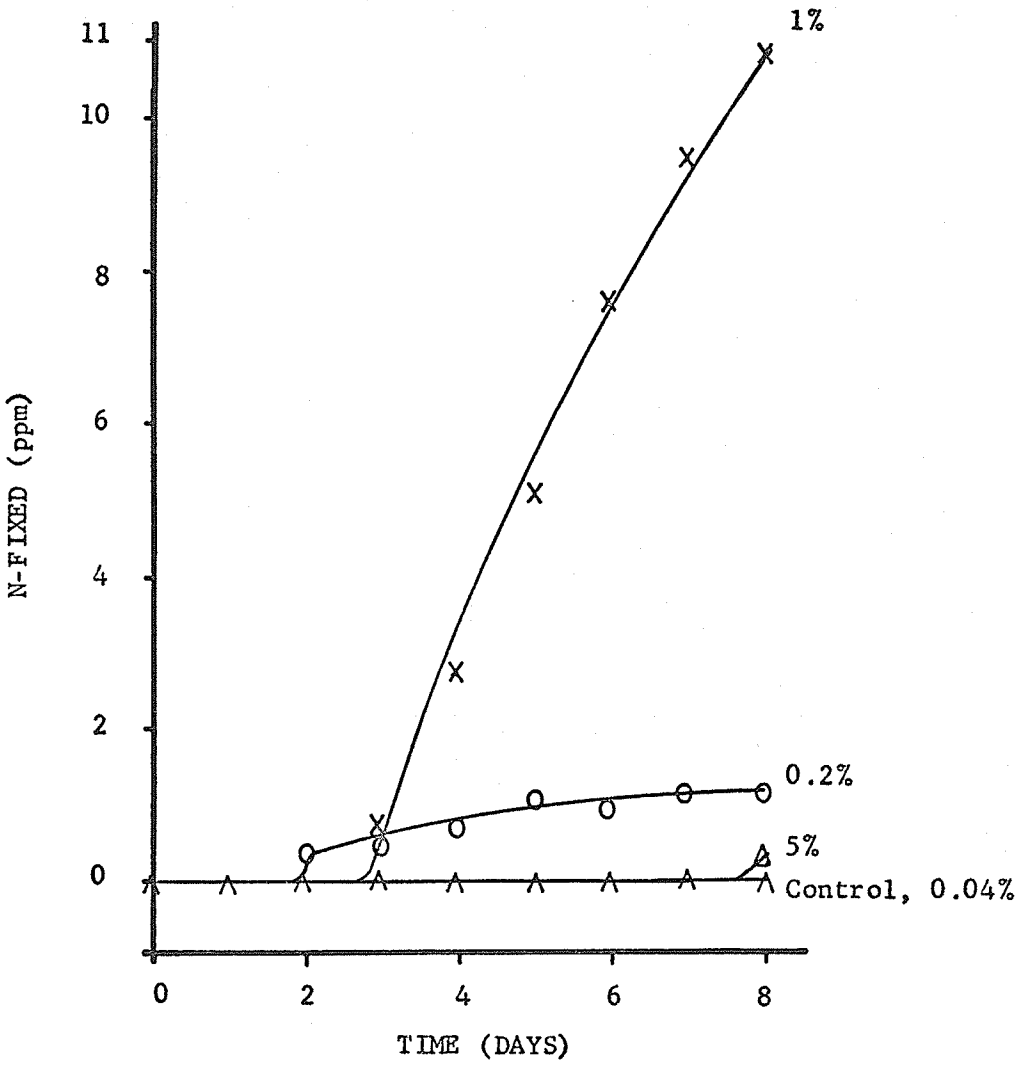

FIG. 13 - RATE OF C_2H_4 PRODUCTION WITH GLUCOSE ADDITION AT 15°C.

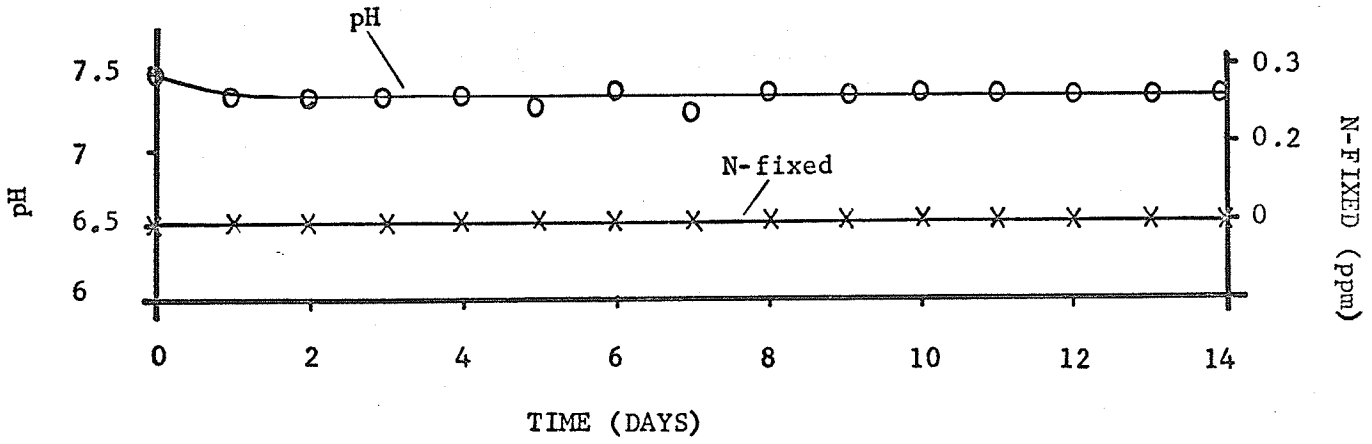

FIG. 14 - RATE OF C_2H4 PRODUCTION AND pH CHANGE WITH ADDED GLUCOSE (5%) AT 15°C.

using 1% glucose, Fig. 9, where pH dropped from 7.5 to 6.5 and there was great fixation of N_2.

If soil pH was used as a means of detecting the microbial activity in the soil, the relative constancy of soil pH and the trace amount of C_2H_4 produced with 5% glucose at 15°C coincides well. The possible cause of the inactivity of microorganisms at this condition was already discussed.

Rate of C_2H_4 production at 25°C.

Acetylene formed at 25°C at various glucose addition is shown in Fig. 15. At this temperature, a measurable amount of C_2H_4 was detected by the second day. A significant amount of C_2H_2 was reduced by samples supplemented with all glucose levels except 0.04%. In every case, the rate was most rapid at the early stages and decreased with time. With 5% glucose no further C_2H_4 production took place after 6 days incubation. This might have been due to lack of oxygen, as suggested by Hardy and Knight(29) when working with nodules. With 1% glucose C_2H_4 production continued through the 14-day incubation period, with decreasing rate at the latter part of the period. Maximum fixation for samples supplemented with 0.2%, 1%, and 5% glucose were approximately 1 ppm, 9.7 ppm, and 10 ppm, respectively, in 14 days.

Rate of C_2H_4 production at 35°C.

The rate of C_2H_4 production (Fig.16), in samples supplemented with 0.04% and0.2% glucose was the same as that of the control sample in which less than 0.1 ppm of C_2H_4 was produced during the 14-day incubation period. Apparently the low glucose concentration supplied was not enough to stimulate measurable C_2H_4 produced. Samples with 1% glucose produced approxi-

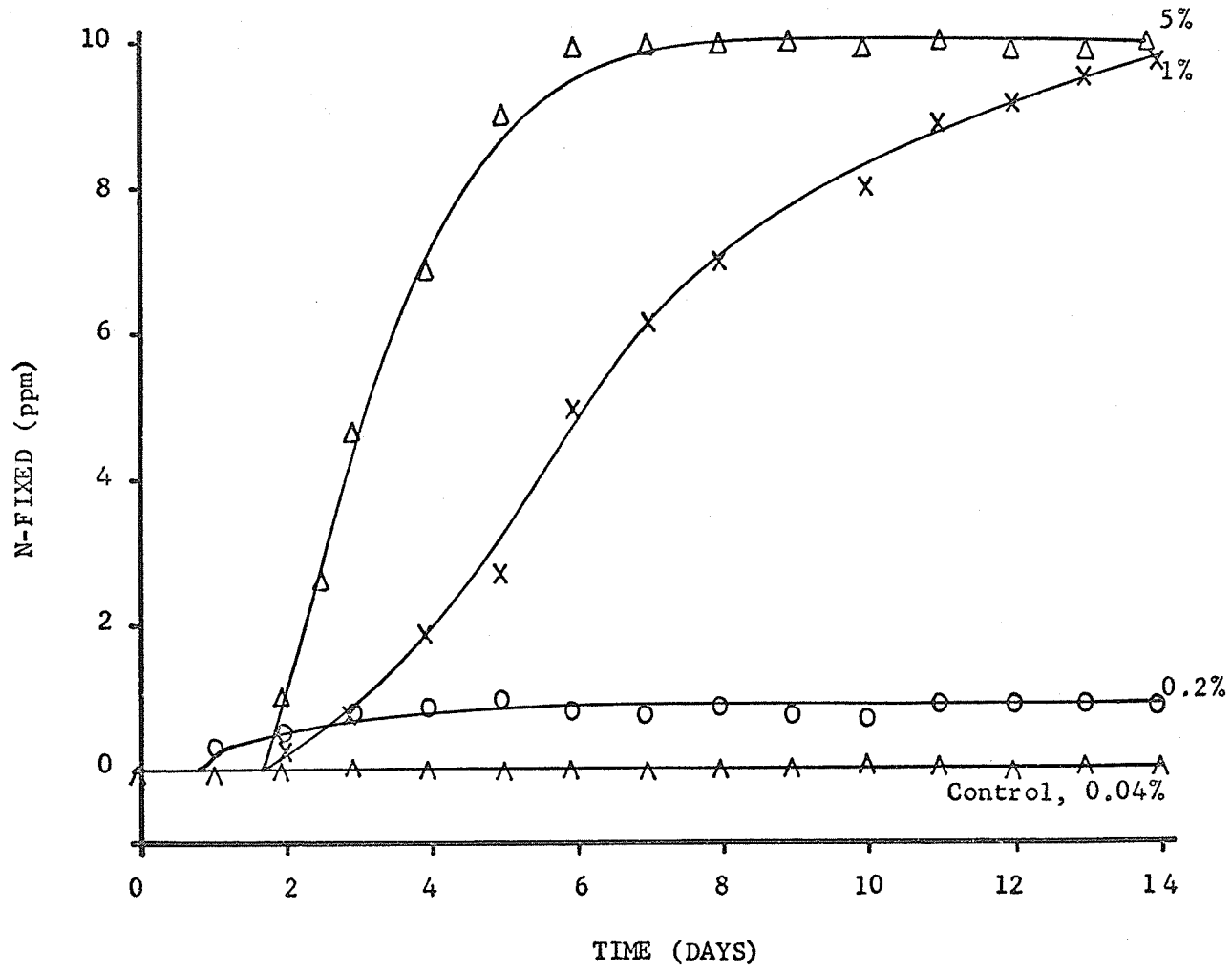

FIG. 15 - RATE OF C_2H_4 PRODUCTION WITH GLUCOSE ADDITION AT 25°C.

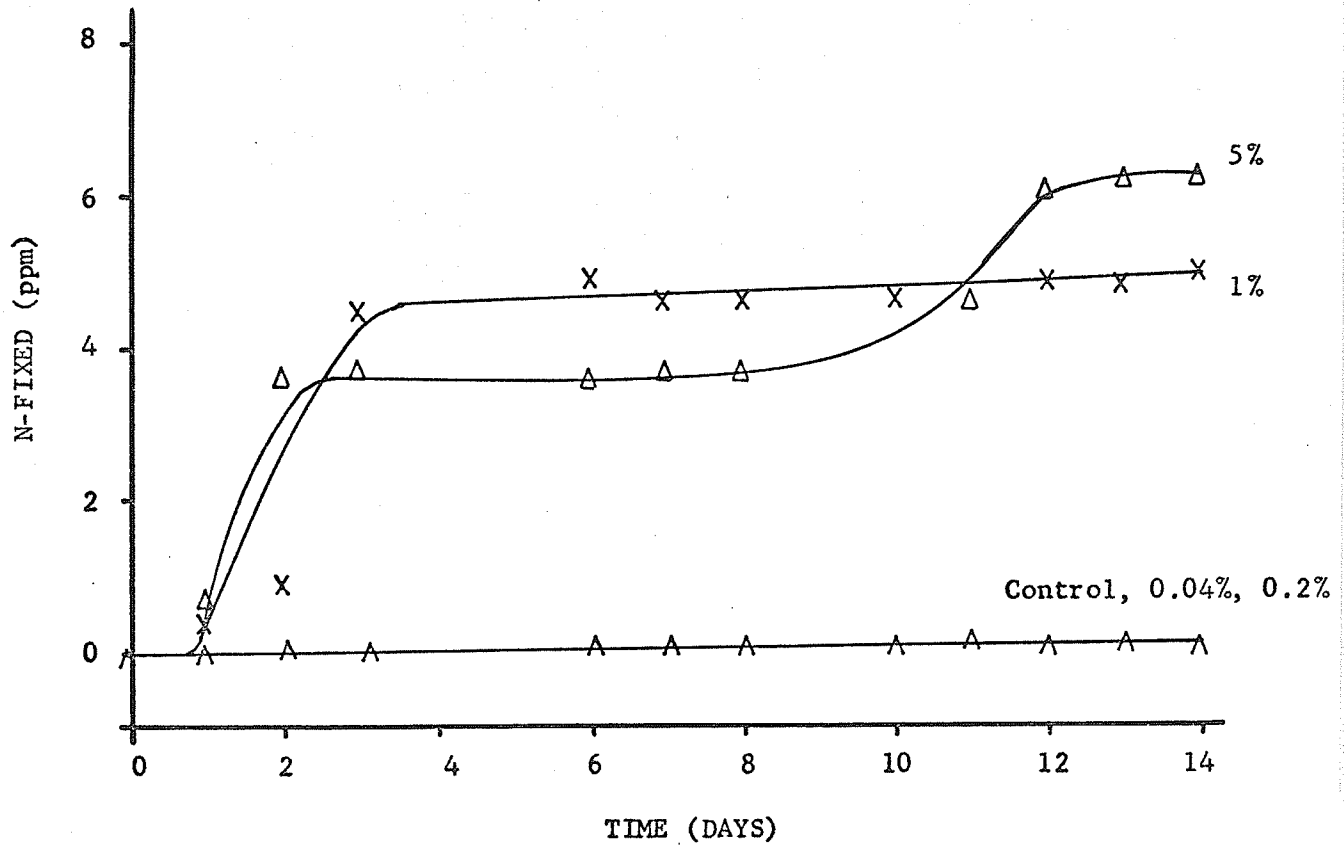

FIG. 16 - RATE OF C_2H_4 PRODUCTION WITH GLUCOSE ADDITION AT 35°C.

mately 4.7 ppm C_2H_4 in the first three days and thereafter C_2H_2 reduction took place very slowly. Samples with 5% glucose reached an initial peak of 3.7 ppm after 3 days and there was very little additional C_2H_4 produced during the next 5 days. The constancy might be caused by the death of the microorganisms which were at stationary or death phase of the bacterial growth curve. With samples containing 5% glucose C_2H_4 production increased again after the 8th day and reached a high of approximately 6.3 ppm on the 14th day. The reason for the sudden increase after the constancy is not known.

Effect of glucose and temperature upon acetylene reduction.

The effect of the addition of glucose at various temperatures upon the C_2H_4 production was studied in incubation experiments lasting for 14 days. The results for the first 8-day incubation period are summarized in Fig. 17. The result of Fig. 17 and the similar experiment of N_2-fixation as shown in Fig. 10 are almost identical. The production of C_2H_4 at 5% glucose addition at low temperature was always least, while maximum value was always obtained at 15°C with 1% glucose addition. These have been discussed already in sections involving ^{15}N tracer method.

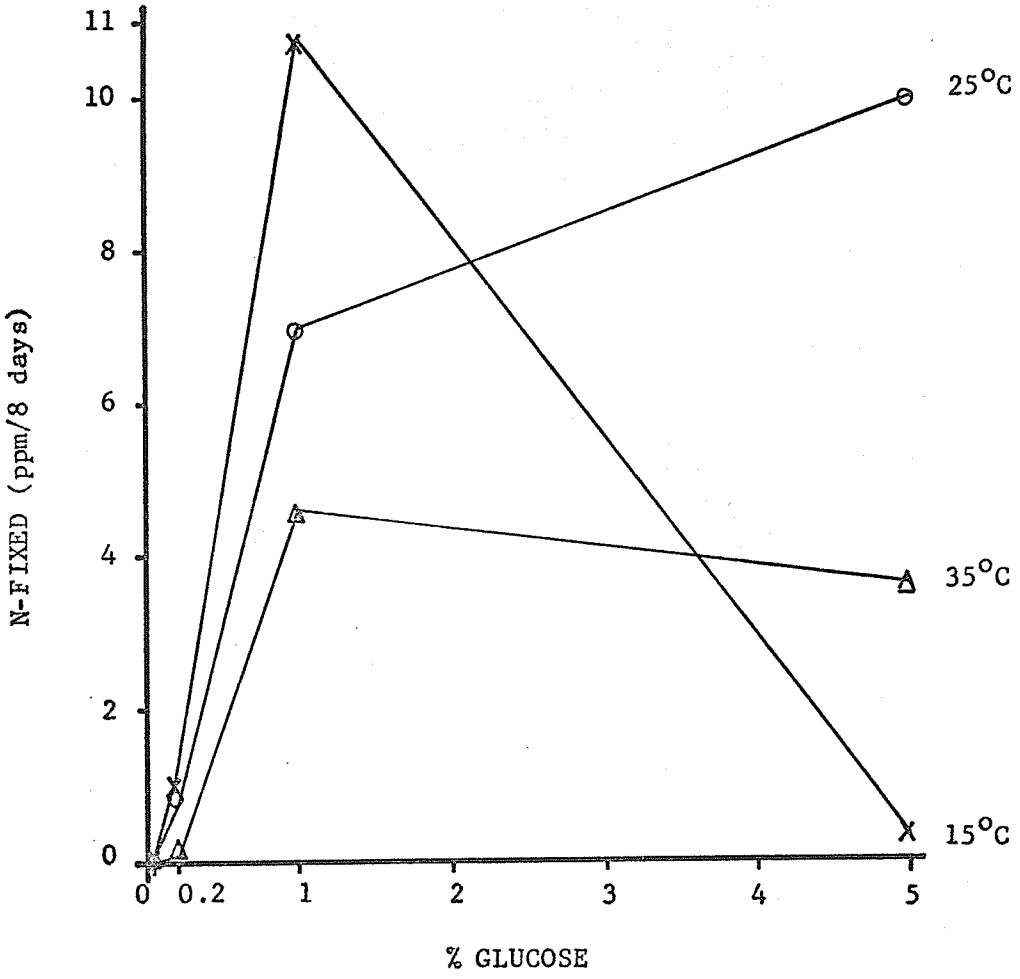

FIG. 17 - EFFECT OF GLUCOSE ON C_2H_4 PRODUCTION AT 3 TEMPERATURES.

GENERAL DISCUSSION

The incubation vessels designed were found to be ideally suited for N-fixation study for a short time period. Aeration by diffusion and by forced aeration can both be carried out with maintenance of constant soil moisture. The amount of total $^{15}N_2$ gas required for this study is relatively small so that the cost of each experiment is low.

The proper concentration of each gas in the artificial atmosphere within the vessel during the study is very important in promoting better growth of nitrogen fixers, and hence a higher N-fixation. In all the investigation here, partial pressure of O_2 was initially brought to 0.16 atm. and this level was not maintained during the incubation period. Thus, after the 8-day or 14-day incubation period, the atmosphere inside the vessel would become less aerobic or more anaerobic. This implied that strict aerobes, facultative aerobes and anaerobes, and strict anaerobes might have contributions to the total N-fixed.

When the data obtained by ^{15}N tracer method and C_2H_2 reduction method were compared (Figs. 10, 17), the trend of the effect of glucose addition and temperatures upon N-fixation appeared very similar. This is in agreement with other workers that the same enzyme system is involved in N-fixation and C_2H_2 reduction. Both methods of analysis indicate that addition of at least 1% glucose or organic material equivalent to 1% glucose to soil and incubated at 15°C is most favorable for N-fixation. There was little or no reduction when samples were supplemented with 5% glucose and incubated at low temperature, 15°C. The most possible cause of low N-fixation at this temperature would be the osmotic

effect in which the increased osmotic pressure exerted by the high glucose concentration, 5%, added to the soil played a great part in retarding the microbial activity. However, this was not the only factor. The main factor was probably a physiological one due to the action of the substance upon the living protoplasm of the cell changing its chemical and physical properties so that it could not function normally.

In every case, irrespective of the method used, N-fixation was increased with the addition of at least 1% glucose at 15°C, 25°C, and 35°C. A temperature of 35°C was beyond the optimum temperature for N-fixation.

Comparing the data of Bremner and Shaw(15) with those obtained here with similar soil characteristics, it was noticed that the addition of 1% glucose in their experiment did not result in a measurable amount of nitrogen being fixed as determined by Kjeldahl total nitrogen method. Using ^{15}N tracer method in this study, the addition of 1% glucose to soil was determined to have approximately 2 ppm per day while approximately 0.9 ppm per day was obtained by C_2H_2 reduction method. This indicates that the two methods used in this study are more sensitive than Kjeldahl total nitrogen method.

The C_2H_2 reduction method used to assay for N-fixation is sensitive, simple, rapid and economical as stated by Hardy and his co-workers(29). The amount of N-fixed converted from C_2H_4 produced is always lower than that obtained by ^{15}N tracer method. One of the possible differences may be due to different solubility of these gases in water and 1% KOH. It may also be due to the use of the conversion factor, 1/3. This factor is a theoretical value in which 3 mole of acetylene reduced was equivalent to 1 mole of nitrogen reduced. This value might be subject to

change depending on the soil type, species of microorganisms, soil depth and temperature. Thus the value of N-fixed deduced from the theoretical equation may not be as quantitative as that obtained by ^{15}N tracer method. If the factor of 1/3 had not been taken into consideration, the value of N-fixed obtained by C_2H_2 reduction method would be three times greater. It is shown in Fig. 18 that the N-fixed by C_2H_2 reduction is approximately 1/4 that of the ^{15}N tracer value at high reduction, and this ratio decreased with decreased incubation period. Therefore the conversion factor for the soil investigated is probably 4/3 instead of 1/3.

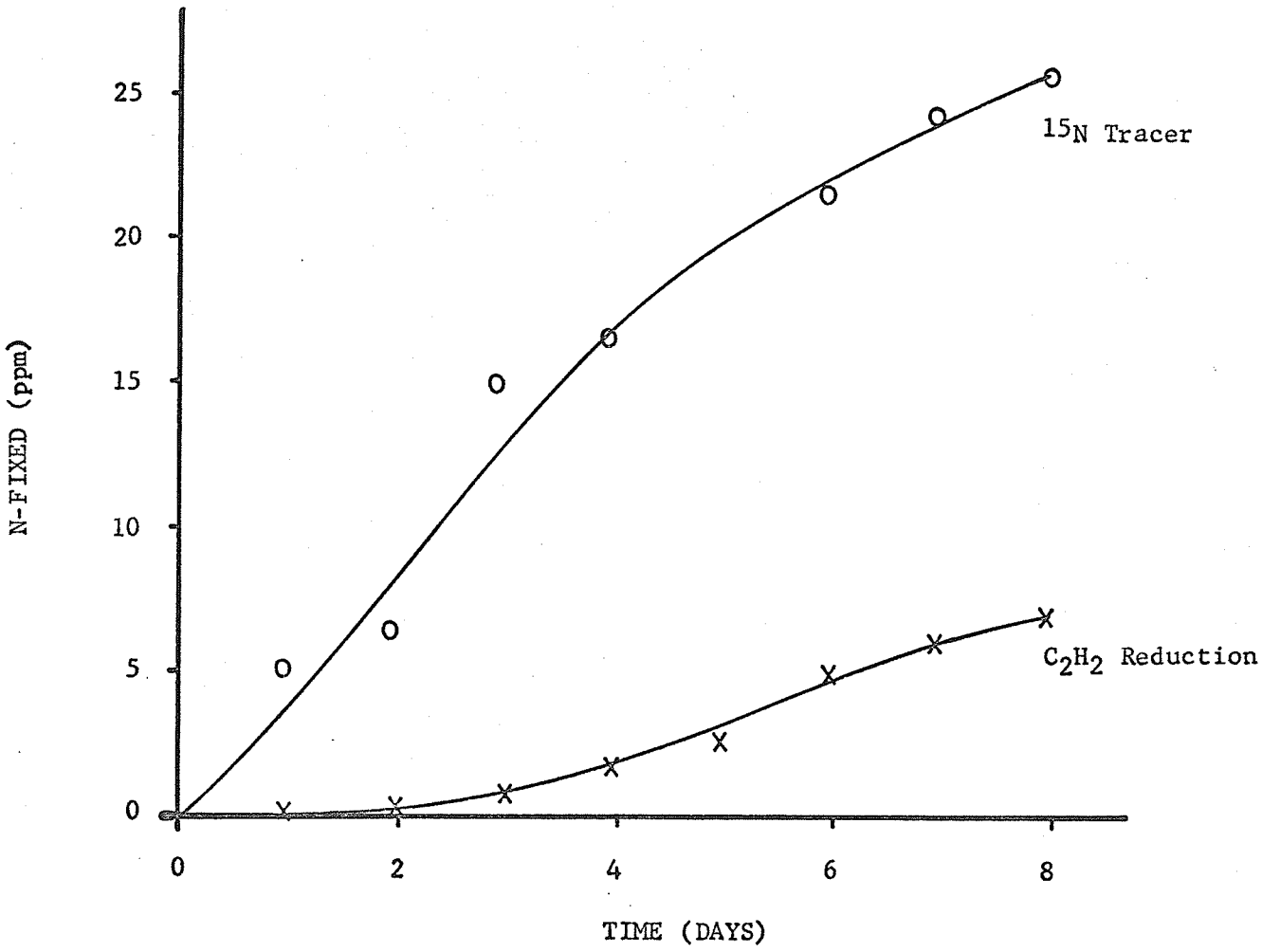

FIG. 18 - RATE OF N-FIXATION AT 25°C, 1% GLUCOSE BY ^{15}N TRACER METHOD AND C_2H_2 REDUCTION METHOD.

SUMMARY

Nitrogen fixation by non-symbiotic process in soil was investigated using $^{15}N_2$ and C_2H_2 reduction methods at several glucose levels, 0.04%, 0.2%, 1%, and 5%, and temperatures, 15°C, 25°C, and 35°C. Incubation vessels for N-fixation study using both techniques were developed and used in this study.

The acetylene reduction method gave good agreement with that of ^{15}N tracer method as regards to the relative amount of nitrogen fixed with different amounts of energy during 8 days. Nitrogen fixation was increased with addition of exogenous energy source up to at least 1% glucose. Temperature of 15°C to 25°C are favorable for nitrogen fixation.

The ^{15}N tracer method for determining N-fixation is expensive and time-consuming, while that of acetylene reduction method is simple, economical, sensitive and less time-consuming, but the calculated value of N-fixed by Portage clay loam using the theoretical equation for C_2H_2 reduction method is about 1/4 that of N-fixed using ^{15}N tracer method.

REFERENCES

1.* Abd-el-malek, Y., and Ishac, Y. Z. 1962. Abundance of Azotobacter in Egyptian soil. Abstr. VIII. Intern. Congr. Microbiol. Montreal. 57.

2. Anderson, G. R. 1955. Nitrogen fixation by Pseudomonas-like soil bacteria. J. Bact. 70:129-133.

3. Becking, J. H. 1961. Studies on nitrogen-fixing bacteria of genus Beijerinckia. I. Geographical and ecological distribution in soils. Plant and Soil. 14:49-81.

4.* Beijerinck, M. W. 1901. Zentr. Bakteriol. Parasitenk., Abt. II. 7:561.

5. Bremner, J. M., and Shaw, K. 1958. Denitrification in soil. I. Method of investigation. J. Agr. Sci. 51:22-39.

6. Bremner, J. M. 1965. Total nitrogen. In Methods of Soil Analysis. p. 1164. Monograph No. 9. Vol. II. Am. Soc. of Agron., Madison, Wisconsin.

7. Bremner, J. M. 1965. Isotope-Ratio analysis of nitrogen in nitrogen-15 tracer investigations. In Methods of Soil Analysis. p. 1257. Monograph No. 9. Vol. II. Am. Soc. of Agron., Madison, Wisconsin.

8. Bremner, J. M., Cheng, H. H., and Edwards, A. P. 1965. Assumptions and errors in nitrogen-15 tracer research, pp. 429-442. In The Use of Isotopes in Soil Organic Matter Studies. Pergamon Press, Toronto.

9. Biggins, D. R., and Postgate, J. R. 1969. Nitrogen fixation by cultures and cell free extracts of Mycobacterium flavum 301. J. Gen. Microbiol. 56:181-193.

10. Brown, M. E., Burlingham, S. K., Jackson, R. M. 1962. Studies on Azotobacter species in soil. II. Populations of Azotobacter in the rhizosphere and effects of artificial inoculation. Plant and Soil. 17:320.

11. Bulen, W. A., Burns, R. C., and LeComte, J. E. 1964. Nitrogen fixation: Cell free system with extracts of Azotobacter. Biochem. Biophys. Res. Commun. 17:265.

12. Bulen, W. A., and LeComte, J. R. 1966. The nitrogenase system from Azotobacter: Two-enzyme requirement for N_2 reduction, ATP-dependent H_2 evolution and ATP hydrolysis. Proc. Natl. Acad. Sci. U.S. 56:979-986.

13. Burk, D., Lineweaver, H., and Horner, C. K. 1934. The specific influence of acidity on the mechanism of nitrogen fixation by Azotobacter. J. Bact. 27:325.

14. Burris, R. H., and Miller, C. E. 1941. Application of ^{15}N to the study of biological nitrogen fixation. Sci. 93:114.

15. Campbell, N. E. R., Dular, R., and Lees, H. 1967. The production of $^{13}N_2$ by 50 Mev. protons for use in biological nitrogen fixation. Can. J. Microbiol. 13:587.

16. Carnahan, J. E., Mortenson, L. E., Mower, H. F., and Castle, J. E. 1960. Nitrogen fixation in cell-free extracts of Clostridium pasteurianum. Biochem. Biophys. Acta. 38:188-189.

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

18. Cheng, H. H., and Bremner, J. M. 1966. Determination and isotope-ratio analysis of different forms of nitrogen in soils: 2. A simplified procedure for isotope-ratio analysis of soil nitrogen. Soil Sci. Soc. of Am. Proc. 30:450-452.

19. Chang, P. C., and Knowles, R. 1964. Non-symbiotic nitrogen fixation in some Quebec soils. Can. J. Microbiol. 11:29-38.

20. Cho, C. M., and Haunold, E. 1965. Some problems encountered in the preparation of nitrogen-15 gas samples and mass spectrometric work. In The Use of Isotopes in Soil Organic Matter Studies. Pergamon Press, Toronto.

21. Delwiche, C. C., and Wijler, J. 1956. Non-symbiotic nitrogen fixation in soil. Plant and Soil. 7:113-129.

22.* Derx, H. G. 1950. A new genus of nitrogen-fixing bacteria occurring in tropical soils. Proc. Kon. Vederl. Akad. Wetensch. 53:140-147.

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

24.* Drewes, K. 1928. Uber die Assimilation des Luftstickstoffs durch Blaualgen. Centralblatt f. Bakt., Par. U. Intektionskrankheiten, 2nd Abt.

25. Dua, R. D., Burris, R. H. 1963. Stability of nitrogen-fixing enzymes and the reactivation of a cold labile enzyme. Proc. Natl. Acad. Sci. U.S. 50:169-175.

26. Grau, F. H., and Wilson, P. Q. 1962. Physiology of nitrogen fixation by Bacillus polymyxa. J. Bact. 83:490-496.

27. Greaves, J. E. 1916. The influence of salts on the bacterial activities of the soil. Soil Sci. 2:443-480.

28. Hardy, R. W. F., and Knight, E. Jr. 1967. ATP-dependent reduction of azide and HCN by nitrogen fixing enzymes of Azotobacter vinelandii and Clostridium pasteurianum. Biochem. Biophys. Acta. 139:69-90.

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

30. Hardy, R. W. F., and Burns, R. C. 1968. Biological nitrogen fixation. Ann. Rev. Biochem. 37:331-358.

31. Hamilton, P. B., and Wilson, P. W. 1955. Nitrogen fixation by Aerobacter aerogenes. Ann. Acad. Sci. Fennicae (A. II) 60:139-150.

32. Jackson, M. L. 1962. Soil Chemical Analysis. p. 47. Prentice-Hall, Inc. Englewood Cliffs, N. J.

33. Jensen, H. L., Petersen, E. J., De, P. K., and Bhattacharya, R. 1960. A new nitrogen-fixing bacterium: Derxia gummosa. Nor. Gen. Nob. Spec. Arch. Microbiol. 36:182.

34. Jensen, H. L. 1965. Nonsymbiotic nitrogen fixation. pp. 430-480. In Soil Nitrogen. Monograph No. 10. Am. Soc. of Agron. Madison, Wisconsin.

35.* Jodin. 1862. Du role physiologique de I'azote (etc). Comp. Rend. Acad. Sci. (Paris) 55:612-615.

36. Johnson, D.D., and Guezi, W. D. 1963. Influence of salts on ammonium oxidation and carbon dioxide evolution from soil. Soil Sci. Soc. Am. Proc. 27:663-666.

37. Katznelson, H. 1940. Survival of Azotobacter in soil. Soil Sci. 49:21.

38. Knowles, R. 1965. The significance of non-symbiotic nitrogen fixation. Soil Sci. Soc. Am. Proc. 29:223.

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

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

41.** Lipman, C. B., and Sharp, L. T. 1912. Toxic effects of "alkali salts" in soils on soil bacteria. III. Nitrogen fixation. In Centbl. Bakt. (etc.). Abt. 2. Bd. 35:647-655.

42. Meiklejohn, J. 1956. Preliminary number of nitrogen fixers on Broadbalk Field. VI Congr. Intern. Sci. Sol. (Paris). 3:243-248.

43. Mortenson, L. E., Valentine, R. C., and Carnahan, J. E. 1962. An electron transport factor from Clostridium pasteurianum. Biochem. Biophys. Res. Commun. 7:448.

44. Mortenson, L. E., Valentine, R. C., and Carnahan, J. C. 1963. Ferredoxin in the Phosporoclastic reaction of pyruvic acid and its relation to nitrogen fixation in Clostridium pasteurianum. J. Bio. Chem. 238:794-800.

45. Mortenson, L. E. 1966. Components of cell-free extracts of Clostridium pasteurianum required for ATP-dependent H_2 evolution from Dithionite and for N_2-fixation. Biochem. Biophys. Acta. 127:18-25.

46. Newton, J. W., Wilson, P. W., and Burris, R. H. 1953. Direct demonstration of ammonia as an intermediate in N-fixation by Azotobacter. J. Biol. Chem. 204:445.

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

48. Parker, C.A. 1954. Non-symbiotic N-fixation bacteria in soil. I. Studies on Clostridium butyricum. Australian J. Agric. Res. 5:90-97.

49. Parker, C. A. 1954. Effect of oxygen on the fixation of nitrogen by Azotobacter. Nature. 173:780-781.

50. Parker, C. A. 1957. Non-symbiotic nitrogen fixing bacteria in soil. III. Total nitrogen changes in field soil. J. Soil Sci. 8:48-59.

51. Parker, C. A., and Scutt, P. B. 1960. The effect of oxygen on nitrogen fixation by Azotobacter. Biochem. Biophys. Acta. 38:230.

52. Paul, E. A., and Newton, D.A. 1961. Studies on aerobic, non-symbiotic nitrogen-fixing bacteria. Can. J. Microbiol. 7:7-13.

53. Pengra, R. M., and Wilson, P. W. 1958. Physiology of nitrogen fixation by Aerobacter aerogenes. J. Bact. 75:21-25.

54. Proctor, M. H., and Wilson, P. Q. 1958. Nitrogen fixation by gram-negative bacteria. Nature. 182:891.

55.* Schloesing, T. 1888. Sur les relations de l'azote atmospherique avec la terre vegetale. Comp. Rend. Acad. Sci. (Paris). 106:898-902.

56. Schollhorn, R., and Burris, R. H. 1966. Study of intermediate in nitrogen fixation. Federation Proc. 25:710.

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

58.* Schmidt-Lorenz, W., and Rippel-Baldes, A. 1957. Wirkung des Sauerstoffs auf Wachstum und Stickstoff bindung von Azotobacter chroococcum, Beijk. Arch. Mikrobiol. 28:45-68.

59. Stewart, W. D. P., Fitzgerald, G. P., and Burris, R. H. 1967. In situ studies on nitrogen fixation using the acetylene reduction technique. Proc. Natl. Acad. Sci. U.S. 58:2071-2078.

60.* Winogradsky, S. 1895. Recherces sur l'assimilation de l'azote libre de l'atmosphere par les microbes. Arch. Sce. Biol. (Petersburg). 3:297-352.

61. Ziemecka, J. 1932. The Azotobacter test of soil fertility applied to classical fields at Rothamsted. J. Agr. Sci. 22:797-810.

* As reviewed by Jensen, H.L. 1965. Nonsymbiotic nitrogen fixation. pp. 430-480. In Soil Nitrogen. Monograph No. 10. Am. Soc. of Agron. Madison, Wisconsin.

**As reviewed by Greaves, J.E. 1916. The influence of salts on the bacterial activities of the soil. Soil Sci. 2:443-480.