AN ABSTRACT OF THE THESIS OF

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Title: A BIOCHEMICAL INVESTIGATION OF THE NITROGENASE SYSTEM FROM SOYBEAN ROOT NODULES

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Abstract approved: Harold J. Evans

The primary purpose of this investigation was to determine the conditions necessary for the preparation of cell-free extracts of nodule bacteroids capable of catalysing the reduction of acetylene to ethylene and nitrogen to ammonia. Initial investigations were conducted to determine whether intact soybean root nodules would reduce acetylene to ethylene and to establish the optimum conditions for the reaction. An extraction procedure for the preparation of cell-free extracts from nodule bacteroids has been developed which involves the use of anaerobic conditions, a buffered ascorbate medium, and insoluble polyvinylpolypyrrolidone. Low-molecular-weight compounds were removed from extracts by use of polyacrylamide gel chromatography. Both acetylene reduction and nitrogen fixation reactions required an ATP-generating system, an electron donor such as $Na_2S_2O_4$, and cell-free extract containing the nitrogenase system. The optimum conditions are reported for catalysis of nitrogen fixation by

these preparations.

Reaction mixtures containing an ATP-generating system, $Na_2S_2O_4$ and nitrogen evolved hydrogen during the course of nitrogen fixation. Under an atmosphere of argon the rate of hydrogen evolution was much greater than under nitrogen. The rate of nitrogen fixation by the bacteroid nitrogenase system was strongly inhibited by hydrogen.

The nitrogenase system from nodule bacteroids was fractionated into two components by use of either protamine sulfate or polypropylene glycol precipitation, followed by chromatography on DEAE-cellulose. Iron and molybdenum were concentrated in one fraction and iron in the other. Combination of fractions resulted in a striking stimulation of activity relative to the activity of individual fractions. The effect of different proportions of the two fractions on specific activities was studied. The ratio of the rates of reduction of acetylene and nitrogen by extracts or fractions of different purities was relatively constant.

A Biochemical Investigation of the Nitrogenase System from Soybean Root Nodules

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ATP	Adenosine-5'-triphosphate
DEAE	Diethylaminoethyl cellulose
K_i	Michaelis constant for inhibition
K_m	Michaelis constant
K_p	Apparent Michaelis constant
Pi	Inorganic phosphate
TES	N-tris(hydroxymethyl)methyl-2-aminomethane sulfonic acid
Tricine	N-tris(hydroxymethyl)methyl glycine
Tris	Tris(hydroxymethyl)aminomethane
V_{max}	Maximum velocity

A BIOCHEMICAL INVESTIGATION OF THE NITROGENASE SYSTEM FROM SOYBEAN ROOT NODULES

INTRODUCTION AND STATEMENT OF PROBLEM

The process of biological nitrogen fixation consists of the reduction of elementary nitrogen to ammonia by living organisms. This biologically fixed nitrogen is the major source of nitrogen available for the formation of protein, which is essential in maintaining the world food supply. The economic importance of biological nitrogen fixation on the Earth cannot be over emphasized. Nitrogen fixation by all biological organisms accounts for 80 percent of the nitrogen fixed on Earth per year (Donald, 1960). Leguminous and non-leguminous symbionts alone account for about 5.5 million tons of nitrogen fixed per year in the United States (Burris, 1965). About 13,000 species of leguminous plants exist, the vast majority of which function as nitrogen fixers. Some of the economically more important legumes include soybean, peas, clover and alfalfa, all of which are infected by different species of Rhizobium.

Prior to 1960, the mechanism of biological nitrogen fixation had been studied to a much less extent than related biochemical areas. The biochemistry of nitrogen fixation was not investigated due to the unsuccessful attempts of numerous investigators to obtain a cell-free extract which contained an enzyme system capable of fixing nitrogen. The preparation of the first cell-free extracts containing consistent

nitrogen fixing ability (Carnahan et al., 1960b), initiated biochemical studies of the nitrogenase system. Since the initial preparation of cell-free extracts containing nitrogenase activity from Clostridium pasteurianum, the enzyme system has been isolated from numerous other free-living bacteria, as well as blue-green algae (c.f. Hardy et al., 1968).

When this study was initiated it had not been possible to obtain an active nitrogenase system from root nodules or from the free-living bacteria which infect the plant, and consequently the biochemical mechanism of symbiotic nitrogen fixation was uncertain. Thus, the primary purpose of this investigation was to prepare cell-free extracts from soybean nodule bacteroids which possessed consistent nitrogen fixing ability. If an active preparation could be obtained it was hoped to perfect a procedure for partial purification of the enzyme system and to investigate its physical properties.

The cell-free nitrogenase systems from Clostridium pasteurianum and Azotobacter vinelandii have been shown to be non-specific for substrate, and will catalyse the reduction of acetylene to ethylene (Shöllhorn and Burris, 1966; Dilworth, 1966). The ability of the nitrogenase system to reduce acetylene was used as a sensitive analytical tool to study the nitrogen fixing system of intact soybean nodules, and to establish optimum conditions for the reaction. The

acetylene reduction technique and the ammonia synthesis assay were used throughout this investigation to study the properties of bacteroid nitrogenase.

REVIEW OF LITERATURE

Numerous reviews on the subject of nitrogen fixation are available, some of which contain extensive historical reviews. Wilson (1963, 1958) has reviewed the early discoveries and the process of non-symbiotic nitrogen fixation. Allen and Allen (1958) have prepared an extensive review of symbiotic nitrogen fixation prior to 1958. Recent reviews concerned with the biochemistry of nitrogen fixation by various organisms includes those by Carnahan and Castle (1963), Mortenson (1965), Burris (1965, 1966), Hardy and Knight (1967), Hardy and Burns (1968), and a book by Stewart (1966).

Early Research on Nitrogen Fixation

In 1838, Boussingault suggested that leguminous plants possess the ability to fix atmospheric nitrogen. This view was not accepted until Hellriegel and Wilfarth (1888) verified Boussingault's conclusion. Winogradsky (1894), working in Russia, demonstrated that certain free-living anaerobic bacteria, such as Clostridium pasteurianum, also possessed the ability to fix nitrogen. At about the same time Beijerinck (1901) found that nitrogen fixation could be accomplished by the aerobic bacteria Azotobacter chroococcum.

During the late 1800's and the early 1900's the study of nitrogen fixation was limited to its practical application, with little emphasis

on its biochemistry. This was due in part to the inability of workers to obtain soluble enzyme preparations capable of fixing nitrogen. Bach, Yermoleva and Stepanian (1939) reported that they had obtained cell-free nitrogen fixation from extracts of Azotobacter chroococcum. However, Burris et al. (1943) were unable to repeat this work using the stable isotope ^{15}N, which is a sensitive method for detecting nitrogen fixation. Continuous efforts by the University of Wisconsin group to obtain cell-free nitrogen fixation with Azotobacter chroococcum using the ^{15}N technique lead to a low degree of fixation in about 25 percent of their experiments (Magee and Burris, 1956). Prior to 1960, numerous other attempts were made to obtain consistent nitrogen fixation by cell-free preparations from a variety of organisms, including Azotobacter vinelandii, Clostridium pasteuranium, Rhodospirillum rubrum, and blue-green algae, and from root nodules (c.f. Carnahan and Castle, 1963). In some cases limited success was achieved, but the methods never yielded reproducible preparations which could be used in studying the biochemistry of nitrogen fixation.

Properties of the Nitrogen Fixing System from Clostridium pasteurianum and Azotobacter vinelandii

In 1960, the first cell-free extracts containing consistent nitrogen fixing ability were prepared from Clostridium pasteurianum by Carnahan et al. (1960a, 1960b). At about the same time Schneider

et al. (1960) developed a method for preparing active cell-free extracts from various blue-green algae, but these extracts lacked the high rate of fixation obtained with Clostridium pasteurianum. Following these initial developments similar success was obtained with Azotobacter vinelandii (Nicholas and Fisher, 1960), Bacillus polymyxa (Grau and Wilson, 1963) and Chromatium (Arnon et al., 1960). Clostridium pasteurianum has been the organism most extensively used in the study of cell-free nitrogen fixation.

Clostridium pasteurianum

Nitrogen fixation by cell-free extracts from Clostridium pasteurianum requires a source of electrons which are provided by the metabolism of pyruvate to acetylphosphate, carbon dioxide, and hydrogen via the phosphoroclastic reaction (Carnahan et al., 1960b). McNary and Burris (1962) suggested that nitrogen fixation by Clostridium extracts required ATP. By replacing pyruvate with an ATP-generating system and an electron donor such as sodium dithionite ($Na_2S_2O_4$) Mortenson (1964) and Hardy and D'Eustachio (1964) demonstrated a positive requirement for ATP in the nitrogen fixing system of Clostridium pasteurianum. In the Clostridium system pyruvate functions as the electron donor and also furnishes ATP via the phosphoroclastic reaction (see Figure 1).

The nitrogen fixing enzyme (nitrogenase) from Clostridium

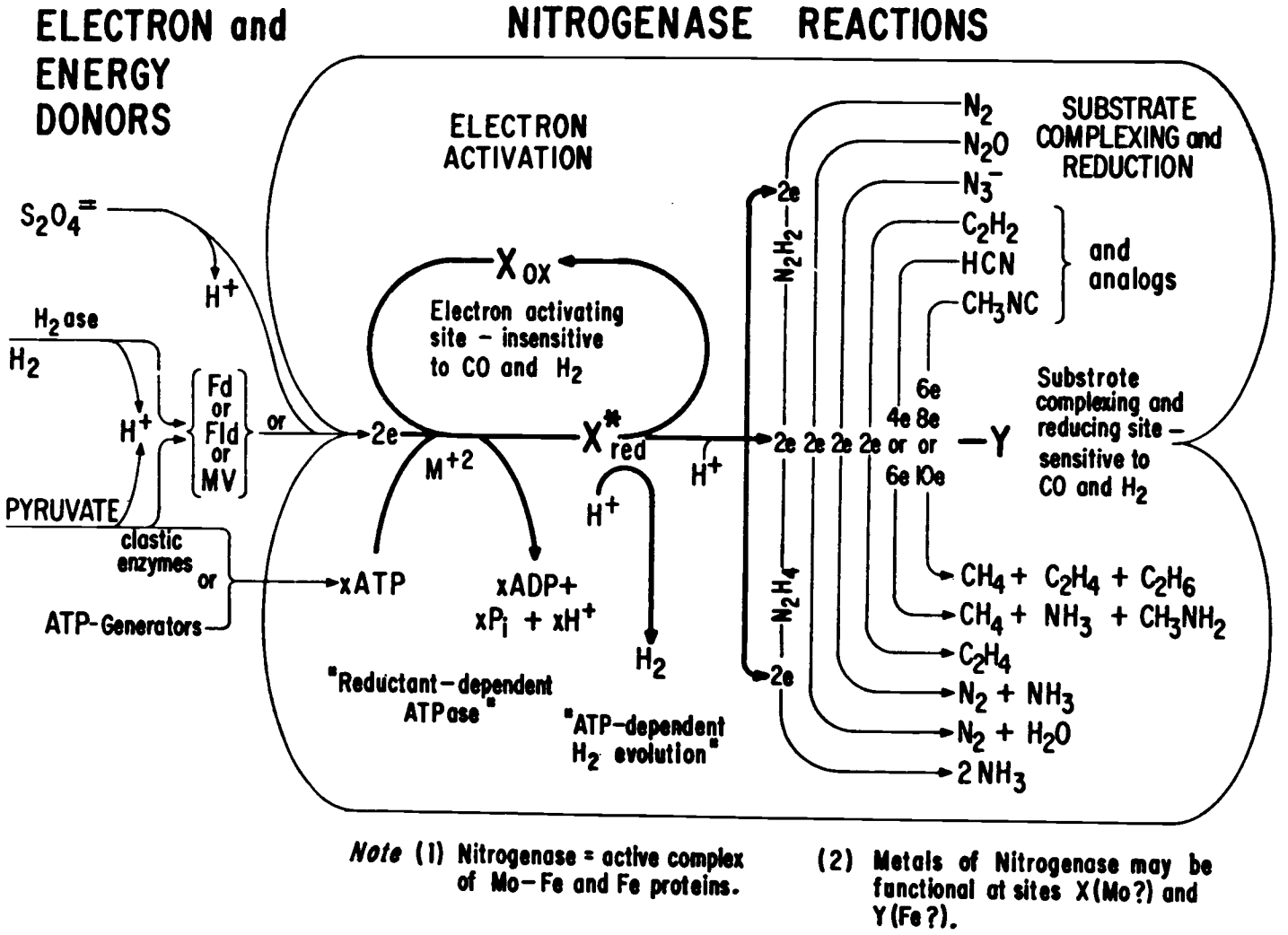

Figure 1. Scheme for nitrogenase and its reactions (c. f. Hardy and Burns, 1968).

pasteurianum (Mortenson, Morris and Jeng, 1967) has been resolved into two components, one component, with a molecular weight of approximately 100,000, that contains molybdenum, non-heme iron, and magnesium, and a second component, with a molecular weight of about 40,000, that contains non-heme iron. The molybdenum-iron component referred to by Mortenson et al. (1967) as molybdoferredoxin was estimated to be 78 percent pure. Both fractions are required for ATP-dependent hydrogen evolution and nitrogen fixation.

In an attempt to isolate the natural electron carrier which functions to transfer electrons from the phosphoroclastic reaction to the nitrogenase system Mortenson, Valentine and Carnahan (1962) isolated a non-heme iron protein from extracts of Clostridium pasteurianum which they termed ferredoxin. Later, Knight, D'Eustachio and Hardy (1966), isolated another protein (flavodoxin) from Clostridium pasteurianum that had been grown on low levels of iron. Bacterial ferredoxin and flavodoxin are the only known natural electron carriers which will function in nitrogen fixation by extracts of Clostridium pasteurianum (see Figure 1). Since the initial isolation of ferredoxin from Clostridium pasteurianum this protein has been isolated from a number of species of Clostridium (Buchanen et al., 1963). Lovenberg et al. (1963) studied the chemical characteristics of ferredoxins from five Clostridium species. These ferredoxins were similar in regard to iron and inorganic sulfide content as well as molecular weight and

electrophoretic character. The differences between the ferredoxins were in spectral properties and amino acid composition. All clostridial ferredoxins studied contained about seven moles each of iron and sulfide per mole of protein, based on a molecular weight of 6000. Realizing the relationship between ferredoxin and the nitrogenase system from Clostridium pasteurianum, Mortenson et al. (1967) have termed the two components of the nitrogenase system molybdoferredoxin and azoferredoxin.

Azotobacter vinelandii

In the initial experiments of Bulen, Burns and LeComte (1965) in which consistent nitrogen fixation by cell-free extracts of Azotobacter vinelandii was demonstrated, a crude extract of Clostridium pasteurianum, lacking nitrogenase but containing ferredoxin and hydrogenase was utilized to transfer electrons from hydrogen gas to the Azotobacter nitrogenase. The hydrogenase enzyme catalyzes the breakdown of molecular hydrogen to hydrogen ion and electrons which are transferred to ferredoxin. Subsequently, Bulen, Burns and LeComte (1965) developed a simplified assay for measurement of nitrogenase activity which utilized sodium dithionite ($Na_2S_2O_4$) as an electron donor and required the addition of an ATP-generating system (see Figure 1). This assay has been used to monitor the progress in purifying the nitrogenase enzyme system from Azotobacter vinelandii

(Bulen and LeComte, 1966). Nitrogenase from this source also was resolved into two components, one containing non-heme iron and molybdenum, and the other containing non-heme iron (Bulen et al., 1966). Both of these components were shown to be essential for nitrogen fixation, ATP-dependent hydrogen evolution and the release of Pi associated with these reactions (Bulen et al., 1966). An investigation (Bulen et al., 1966) of the stoichiometry of the reaction revealed that five molecules of ATP were needed for each electron pair transferred.

Kelly, Klucas, and Burris (1967) also fractionated the nitrogenase enzyme system from Azotobacter vinelandii into two components, and described a method of storage, whereby the activity of the purified fractions may be maintained for long periods of time. This method, which involves freezing and storing the preparations in liquid nitrogen, greatly facilitated further purification of the fractions.

Hardy and Knight (1966) have utilized cell-free extracts of Azotobacter vinelandii and Clostridium pasteurianum in a study of the specificity of the nitrogen fixing enzyme system for substrate. Nitrogenase from these organisms not only catalyzes the reduction of nitrogen to ammonia, but also catalyzes the reduction of N_2O to ammonia, of azide to nitrogen and ammonia and of cyanide to methane and ammonia. In the latter reduction methylamine has been identified as an intermediate. Furthermore, Shöllhorn and Burris (1966), and Dilworth (1966) have shown that nitrogenase from Clostridium

pasteurianum catalyzes the reduction of acetylene to ethylene. The evidence indicating that the nitrogen fixing enzyme is non-specific for electron acceptor and catalyzes the reduction of several compounds other than nitrogen gas is convincing.

Recently Benneman et al. (1969) have described an electron carrier from Azotobacter, referred to as azotoflavin, that can function in the transfer of electrons from photosystem I of spinach chloroplast fragments to nitrogenase isolated from Azotobacter. Photochemical reduction of acetylene in this system was dependent upon an ATP supply. Azotoflavin appears to be identical with the flavoprotein isolated and crystallized from Azotobacter vinelandii by Hinkson and Bulen (1967). By use of the chloroplast fragment assay, Yoch et al. (1969) have identified a ferredoxin compound in Azotobacter vinelandii that also functions in the transfer of electrons from photosystem I to Azotobacter nitrogenase. This Azotobacter ferredoxin contains about six moles each of iron and inorganic sulfide per mole of protein based on a molecular weight of 20,000. Yoch et al. (1970) have reported that a non-heme protein, similar in some respects to ferredoxin, can be isolated from extracts of soybean nodule bacteroids which will function in the transfer of electrons from photosystem I to the Azotobacter nitrogenase.

Symbiotic Nitrogen Fixation

It has been firmly established, by use of the ^{15}N technique (Aprison and Burris, 1952) that excised soybean nodules fix atmospheric nitrogen for a period of about two hours after excision. The rate of fixation is greatly reduced in sliced nodules, and fixation is virtually lost when nodules are crushed (Aprison, Magee and Burris, 1954). Bergersen (1960), using ^{15}N reported that nitrogen fixation in leguminous nodules occurred on a membrane surface surrounding the bacteroids. Klucas (1964) attempted to verify Bergersen's findings, but always found more ^{15}N concentrated in the soluble fraction than in membrane fractions. More recently, Bergersen and Turner (1967) have firmly established that washed suspensions of bacteroids from soybean nodules retained a capacity to reduce nitrogen, indicating that nitrogen fixation occurs within the bacteroid. Bergersen's results, indicating the bacteroid as the site of nitrogen fixation were subsequently verified independently by the author and associates as is indicated in another section of this thesis. Bergersen et al. (1967) also showed that nitrogen fixation by washed bacteroids was stimulated by the addition of oxidizable substrates including succinate, fumarate and pyruvate. Bergersen (1962, 1966) has investigated the effects of partial pressures of oxygen on nitrogen fixation in excised soybean nodules, and has devised a method for the demonstration of nitrogen

fixation in breis of nodules. In his procedure it was necessary to exclude all traces of oxygen while nodules were crushed in a press, but oxygen was an essential component for nitrogen fixation in reaction mixtures containing nodule breis. Bergersen (1966) used the sensitive ^{15}N technique for the detection of nitrogen fixation but obtained no more than 6 mμ atoms of nitrogen fixed per mg of brei protein. The activity of this system was independent of exogenous reductant and an ATP-generating system. At the time these experiments were conducted no reproducible fixation of nitrogen gas by cell-free extracts of leguminous nodules had been reported in the literature.

Leghemoglobin, a heme-containing red pigment, is always present in root nodules of leguminous plants when nodules are active in symbiotic nitrogen fixation. Numerous researchers (Virtanen, Erkama, and Linkola, 1947; Bergersen, 1961) have demonstrated a positive correlation between the rate of nitrogen fixation and the volume of leghemoglobin. Early work suggested that the key step in nitrogen fixation occurred with a valence change of the heme or some other metalloenzyme. Bergersen et al. (1967) have demonstrated the localization of nitrogen fixation within the bacteroid, which excludes a direct role of leghemoglobin in the biochemical process of nitrogen fixation by bacteroids. However, since leghemoglobin occurs only in nitrogen fixing nodules, it might be assumed that leghemoglobin has some role in the overall process of nitrogen fixation. Abel, Bauer

and Spence (1963) claimed to have isolated a ferro-leghemoglobin-nitrogen complex where the heme group is complexed with nitrogen. Appleby (1969) has challenged the existence of this leghemoglobin-nitrogen complex, and has shown that it may only be a physical mixture of leghemoglobin and cytochrome c.

MATERIALS AND METHODS

Chemicals

Reagent grade chemicals or the highest grade available were obtained from commercial sources. Creatine phosphate, creatine phosphokinase, sodium-ATP and tris (hydroxymethyl) aminomethane (tris) were obtained from Sigma Chemical Co., (St. Louis, Mo.). Sodium chloride, magnesium chloride, sodium dithionite ($Na_2S_2O_4$), potassium phosphate, and calcium carbide (for acetylene generation) were obtained from J. T. Baker, (Phillipsburg, N. J.). The polypropylene glycol (P-400), sodium ascorbate and trichloroacetic acid were obtained from Matheson, Coleman and Bell, (Cincinnati, Ohio). The prepurified nitrogen, hydrogen and argon were obtained from National Cylinder Gas, (Portland, Oregon), and traces of oxygen were removed from the gases by the method of Lux (1959). N-tris (hydroxymethyl) methyl-2-aminomethane sulfonic acid (TES) was obtained from Calbiochem, (Los Angeles, Calif.). The protamine sulfate was obtained from Eli Lilly Corp., (Indianapolis, Ind.), and polyvinylpolypyrrolidone (Polyclar AT) obtained from GAF Corp., (San Francisco, Calif.). Isotopic nitrogen (95% ^{15}N) was purchased from the Isomet Corp., (Palisades Park, N. J.). The sodium dimethyl arsonate (sodium cacodylate) was obtained from Mann Research Laboratories, Inc., (New York, N. Y.).

Source of Nodules

Soybean plants (Glycine max Merr. var. Merrit or Chippewa) inoculated with a commercial strain of Rhizobium japonicum (kindly supplies by Dr. Joe Burton of Nitragen Co.) were cultured in a greenhouse or growth chambers in pots of perlite supplied with a nitrogen-free nutrient solution (Ahmed and Evans, 1960). The perlite was sterilized in an autoclave at 15 pounds per square inch for four hours before being utilized. Pots were flushed twice daily with nutrient solution and every fourth day with water to remove any accumulated salts. After 35 to 40 days under 16 hour day lengths the plants were removed from the culture vessels, and the nodules harvested and washed with cold tap water. A typical harvest, which consisted of between 100 and 150 pots containing about 10 soybean plants per pot, yielded a total of approximately 350 gm of nodules. In experiments conducted on intact nodules, or nodule breis, the nodules were stored in ice for no longer than two hours before use. When the nodules were to be used for the preparation of cell-free extracts they were either used within one hour after excision, or were frozen in liquid nitrogen and stored at -70° C for use at a later time.

Assays

Ammonia and Ethylene

Nitrogen fixation and acetylene reduction were carried out in 21 ml bottles fitted with rubber serum caps. In experiments conducted on intact nodules and nodule breis the materials to be assayed were placed in reaction vessels, which were then fitted with serum caps, flushed with argon, and filled with the desired gas mixture. In experiments with cell-free extracts, reaction bottles initially contained all reagents except $Na_2S_2O_4$ and nitrogenase. The reaction vessels were fitted with serum caps, flushed three times with argon and filled with the desired gas mixture. The desired amount of nitrogenase was then added with a gas-tight hypodermic syringe, followed by the addition of $Na_2S_2O_4$ to initiate the reaction. Gas samples of less than 0.25 atm were added to the reaction vessels with a syringe after evacuation and addition of other gases. After incubation with shaking in a water bath at 30°C, reactions involving the reduction of nitrogen gas were terminated by the injection of 2 ml of saturated K_2CO_3 solution. In experiments in which acetylene reduction was measured the reactions were terminated with the addition of 0.5 ml of 15 percent trichloroacetic acid.

In the assays for nitrogen fixation or acetylene reduction with cell-free extracts the complete reaction mixtures varied in volume

from 1.5 ml to 6.0 ml (see legends of figures and tables). A typical reaction mixture in a final volume of 3 ml contained the following in micromoles: phosphate buffer at pH 7.0, 50; Na_2ATP 15; creatine phosphate, 150; magnesium chloride, 10; sodium dithionite ($Na_2S_2O_4$), 20. In addition this reaction mixture contained 0.8 mg creatine phosphokinase and an appropriate amount of protein (nitrogenase).

The production of ethylene from acetylene was measured by use of an Aerograph 600D gas chromatograph equipped with a hydrogen flame detector and a recorder and integrater. The conditions employed in the chromatograph were as follows: A stainless steel column 150 cm in length and 0.16 cm in inside diameter was packed with 80 to 100 mesh Porapak R. Nitrogen was used as the carrier gas and was passed through the column at a flow rate of 25 cc per minute. The temperature of the column was maintained at 45°C with the injection chamber slightly higher. The ethylene and acetylene peaks from the column were identified by comparison of retention times with those of known standard samples of gas. The identity of the gases also was indicated by the demonstration that the addition of bromine water to a typical reaction mixture resulted in the disappearance of peaks attributed to ethylene and acetylene. Nitrogen fixation was assayed by the method of Dilworth et al. (1965) in which ammonia is distilled into 1 N sulfuric acid, and after two hours the amount of ammonia is estimated colorimetrically by the use of Nessler's reagent (Umbreit,

Burris and Staufer, 1964). The ammonia produced in ^{15}N experiments was collected by microdiffusion into 2 ml of 0.01 M HCl in the center compartment of a Conway dish. The procedure of Mortenson (1961) was followed with the exception that HCl replaced H_3BO_3. After incubation for 14 hours the HCl containing NH_4^+ was removed quantitatively from the center compartment of each dish and diluted with 200 mg of N in the form of reagent grade NH_4Cl. Analyses for ^{15}N were conducted mass spectrometrically (Burris and Wilson, 1957) by Dr. Robert Klucas in the laboratory of Dr. R. H. Burris, and results were corrected for dilution.

Hydrogen Evolution

Manometric techniques were utilized in determining the amount of gas exchange. All components in the reaction except $Na_2S_2O_4$, extract, and KOH were placed into the main compartment of the Warburg flasks. A filter-paper wick and 0.2 ml of 20 percent KOH were placed in the center well. Flasks attached to manometers were placed in the Warburg apparatus at 30°C, flushed with nitrogen or argon, and shaken during an equilibration period of 15 minutes. The enzyme was injected with a hypodermic syringe into the side arm of the vessel through a serum cap, then was tipped into the major compartment. Gassing was continued for three minutes, when the $Na_2S_2O_4$ solution was injected into the side arm, and then tipped into

the main compartment. After five minutes, flasks were closed and pressures recorded. The rate of gas evolution during the first measured increment (5-10 minutes) was extrapolated to zero time. This procedure was employed because of the initial pressure change that occurred immediately after tipping the $Na_2S_2O_4$ solution into the main compartment. Presumably this was due to changes in vapor pressure from mixing reagents or to a release of decomposition products of $Na_2S_2O_4$. The initial change in pressure that occurred immediately after tipping $Na_2S_2O_4$ into the reaction was not enzyme-dependent.

Protein Determination

After precipitating the protein material by the addition of one ml of 15 percent trichloroacetic acid to 0.1 to 0.5 ml of extract, the solution was centrifuged at 500 x g for ten minutes and the supernatant discarded. The protein content of the pellet was then estimated by use of the biuret reagent of Gornall, Bardawill and David (1949). In some experiments the protein content of the extracts were estimated from nitrogen determinations using the micro-Kjeldahl method (Umbreit, Burris and Stauffer, 1964).

Iron and Molybdenum Determination

After one ml aliquots of the extract were digested with concentrated H_2SO_4 and a 30 percent H_2O_2 solution as described by Bulen and LeComte (1966), iron content was determined colorimetrically by the use of o-phenanthroline (Ballentine and Burford, 1957) and molybdenum by the dithiol procedure (Clark and Axley, 1955).

Preparation of Nodule Breis

It was found in preliminary experiments that nodule breis prepared under aerobic or anaerobic conditions, and in buffers of various types, catalyzed little or no acetylene reduction. These nodule breis contained large quantities of green to brown pigments, which, presumably, were phenolic compounds. Phenolics and their oxidation products are known to inactivate many enzymes during extraction from plant tissues (Loomis and Battaile, 1966). Loomis and Battaile (1966) have shown that an insoluble preparation of polyvinylpolypyrrolidone, (PVP) effectively removed phenolic compounds from plant extracts prepared in a buffered ascorbate solution, and thus prevented phenolic oxidation products from inactivating certain plant enzymes. These procedures were incorporated into a method for the preparation of nodule extracts for use in acetylene and nitrogen reduction experiments.

All steps in the preparation of breis and cell-free crude extracts

were performed under anaerobic conditions. This was accomplished by sparging buffers or other reagents with prepurified nitrogen or argon (Lux, 1959), by transferring solutions with hypodermic syringes and by carrying out certain operations in a glove-box filled with nitrogen.

In a typical experiment 150 g of either fresh or frozen nodules, 50 g of insoluble acid-washed polyvinylpolypyrrolidone, and 400 ml of 0.02 M potassium phosphate buffer containing 0.2 M sodium ascorbate at pH 7.4 were placed in the vessel of an Omnimixer (Ivan Sorvall and Co.) fitted with gassing vents. The contents of the vessel were flushed with nitrogen for ten minutes at room temperature following which the nodules were macerated by blending for five minutes at a setting of 50 volts on a variable transformer. The macerated mixture was squeezed through four layers of cheesecloth and the filtrate centrifuged at 7000 g for ten minutes. The supernatant was discarded, and the pellet containing the bacteroids was mixed with 80 ml of 0.025 M tris buffer at pH 7.4. After centrifuging at 7000 g for ten minutes, the pellet was suspended in 20 ml of the respective buffers specified in the legends of figures and in the text.

Preparation of Cell-free Extracts

Various buffers were used in the preparation of cell-free bacteroid extracts including potassium phosphate, potassium cacodylate, tris and TES, with no apparent differences in their effect on the

extracts. Some nodules contained an exceedingly active poly-β-hydroxybutyrate depolymerase which produced β-hydroxybutyric acid, and thus a sharp decrease in pH of the extracts. This decrease in pH was prevented by the addition of 0.1 M tris or 0.05 M TES buffers.

Suspended bacteroid cells (breis) were ruptured under anaerobic conditions with an Aminco French press at a pressure of 16,000 pounds per square inch. The ruptured cells were collected under a stream of nitrogen and centrifuged at 0°C to 2°C at 48,000 x g for 50 minutes with an Ivan Sorvall RC2B refrigerated centrifuge. The supernatant solution (crude extract) was used within two hours or was injected into a 50 ml polyethylene vial containing liquid nitrogen and subsequently stored in a Dewar of liquid nitrogen. A portion of the frozen extract was chipped from the vial, placed into a serum bottle, then capped and flushed with argon and allowed to thaw.

Purification of Nitrogenase

In certain experiments conducted to examine some of the properties of bacteroid nitrogenase, endogenous ammonia and other low-molecular-weight compounds were removed from the crude extract by use of a column of polyacrylamide gel (Bio-gel P2, Bio-Rad Labs, Richmond, Calif.) 3.5 cm in diameter and 15 cm in length. The chromatography was carried out in a glove-box filled with nitrogen. Buffers and the column were saturated with nitrogen prior to use.

About 15 ml of crude extract was placed on the column and eluted with 0.02 M potassium phosphate buffer at pH 7.0. The brown eluate (referred to as Bio-gel extract) was collected and utilized for the various experiments as indicated in the legends of tables and figures. Extracts prepared by this procedure contained 0.04 to 0.06 μmoles of ammonia per mg of protein. In contrast, crude extracts contained 0.2 to 0.4 μmoles of ammonia per mg of protein.

In other experiments performed in an attempt to purify the crude bacteroid nitrogenase two different types of fractionations were conducted prior to DEAE-cellulose chromatography. One fractionation (see Results and Discussion) consisted of precipitating the nitrogenase fraction with polypropylene glycol P-400. The other method (see Figures 13 and 14) consisted of either treating the crude extract with protamine sulfate, followed by heating to remove various protein materials, including nucleoproteins or only treating the crude extract with protamine sulfate.

The final purification step was performed on columns of DEAE-cellulose (Whatman DE-32) using anaerobic techniques essentially the same as those described by Munson, Dilworth and Burris (1965) and Kelley, Klucas and Burris (1967). The DEAE-cellulose was washed successively with 0.5 N HCl, and 0.5 N KOH (15 volumes of each for each weight of dry DEAE-cellulose) and then with sufficient distilled water to bring the pH to near neutrality. Columns were equilibrated

with either 0.05 M TES at pH 8.0, or 0.025 M tris-Cl at pH 7.4 (see Figure 12 and Results and Discussion). The reservoirs of buffers for eluting the column were sparged with high-purity nitrogen prior to use, and a trace of $Na_2S_2O_4$ was added to all buffered salt solutions just before the chromatographic procedure was initiated. The temperature of the columns was maintained at 9°C by use of an external water jacket connected to a temperature-controlled water bath. The extract was layered on the surface of the anaerobic column with a hypodermic syringe, and 10 ml fractions were collected through a hypodermic needle injected into rubber-capped serum bottles maintained anaerobic by a stream of high-purity nitrogen flowing through the bottle. The flow rates of the columns were about 60 ml per hour.

RESULTS AND DISCUSSION

Acetylene Reduction by Intact Nodules

The conditions for optimum ethylene production by soybean nodules are presented in Figures 2-4. A time-course for the production of ethylene from acetylene is illustrated by the data presented graphically in Figure 2. Ethylene production proceeded almost linearly for a period of about one hour, then the rate diminished rapidly and virtually ceased after an incubation period of two hours. Control reaction mixtures containing nodules that previously had been boiled for three minutes produced no ethylene from acetylene. The time-course for acetylene reduction is similar to the time-course of nitrogen fixation by soybean nodules reported by Aprison and Burris (1952).

Sprent (1969) has shown that nodules previously immersed in water are restricted in the length of time they will fix nitrogen due to a reduction in the oxygen supply. The capacity to reduce acetylene can be restored by regassing the nodules with 0.8 atm of oxygen and shaking. The data in Figure 3 show that oxygen is indispensible for the reduction of acetylene to ethylene by soybean nodules. The rate of acetylene reduction proceeded almost linearly at oxygen concentrations between 0.0 and 0.2 atm. The addition of oxygen at concentrations greater than 0.2 atm resulted in no further increase in the rate of

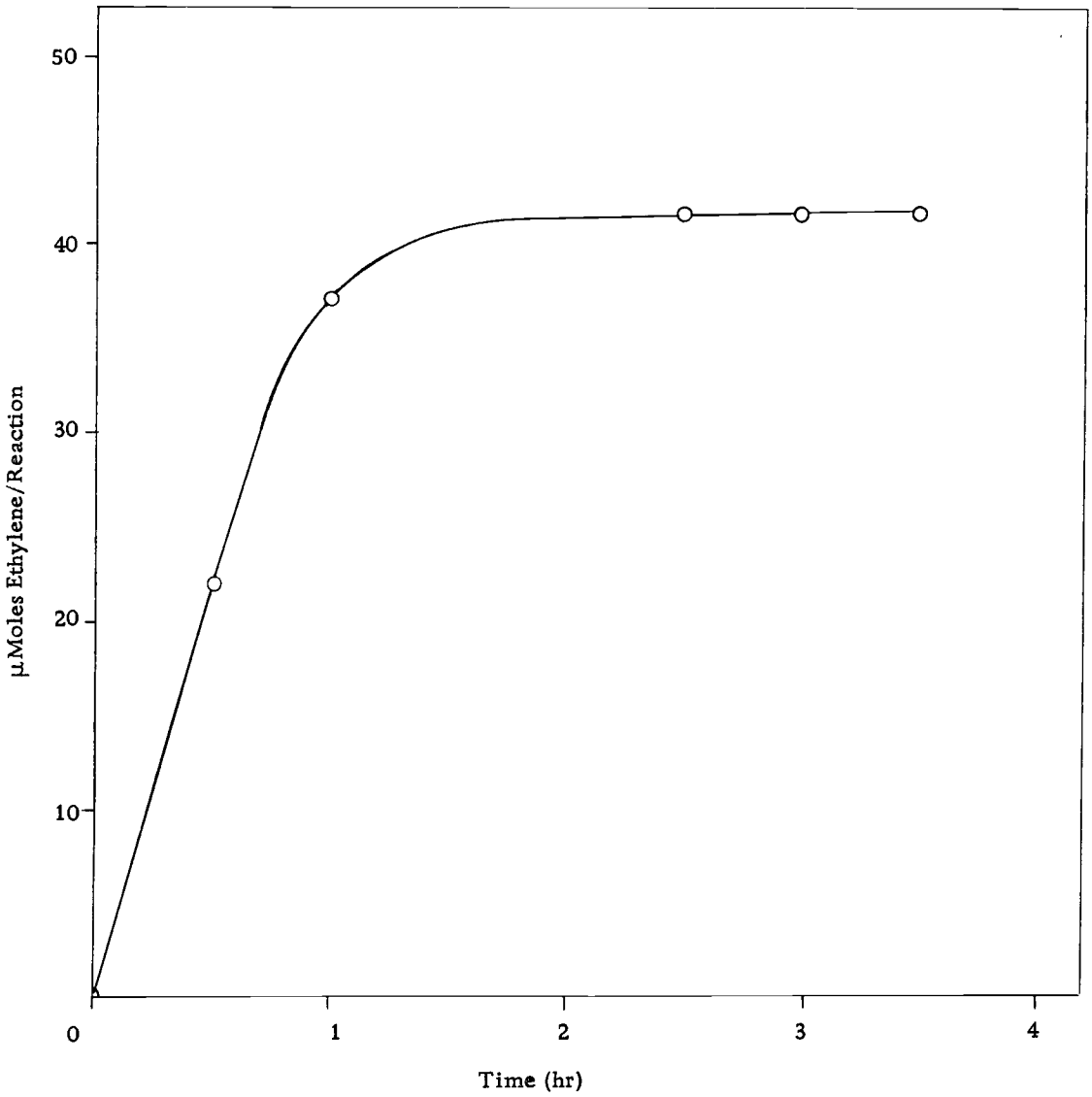

Figure 2. The reduction of acetylene to ethylene by soybean nodules during different periods of incubation. A sample of 5 gm freshly harvested soybean nodules from plants 40 days old were placed in each of six bottles of 21 ml capacity. Bottles were fitted with a rubber serum cap, evacuated and flushed three times with argon then evacuated and filled with 0.25 atm O_2, 0.10 atm of acetylene and 0.65 atm of argon. Reaction mixtures were incubated in a water bath at 30°C for periods of time indicated. Gas samples of 0.5 ml were withdrawn with a syringe and analyzed for ethylene.

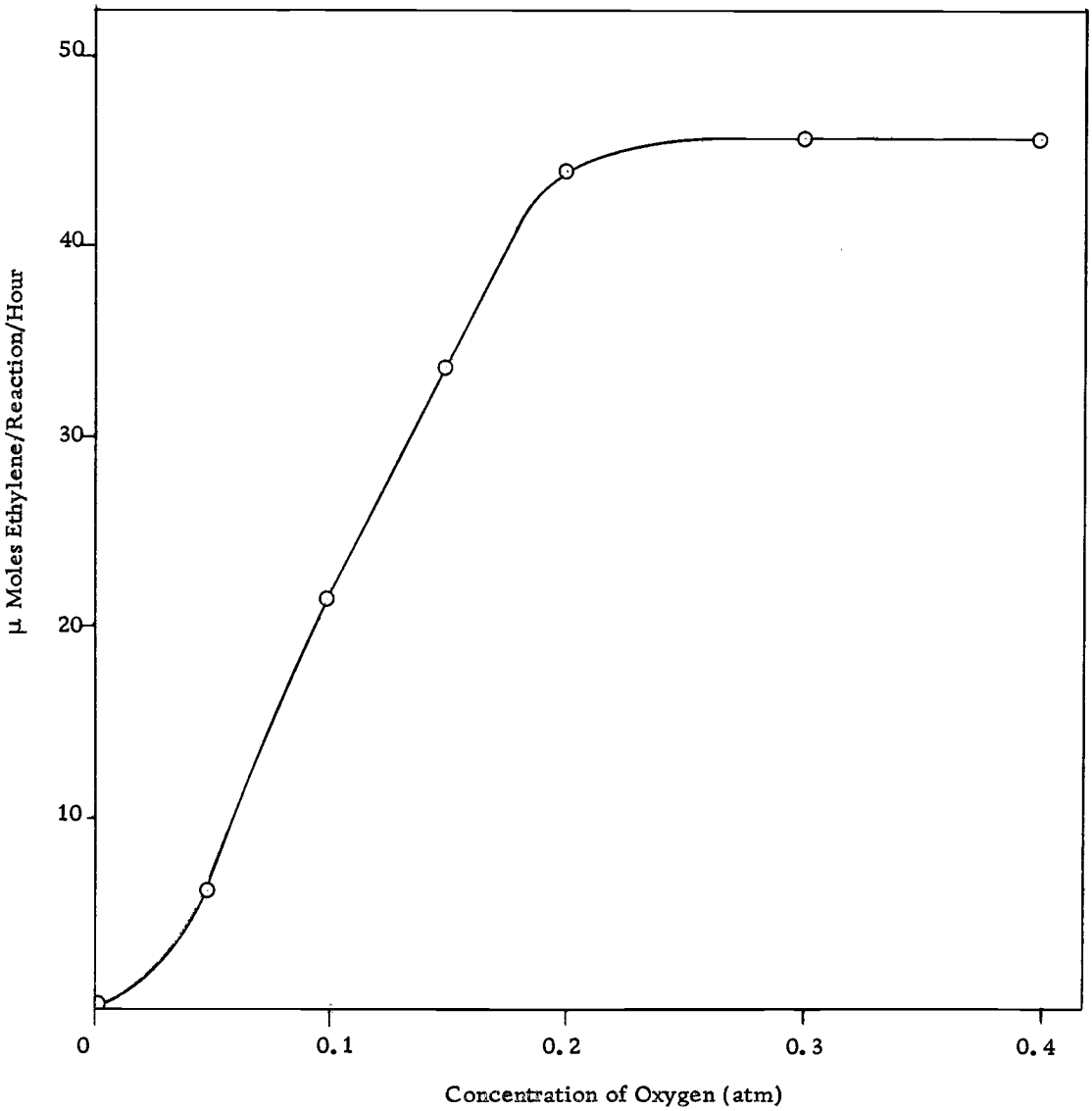

Figure 3. The influence of oxygen concentrations on the reduction of acetylene to ethylene by soybean nodules. The experimental conditions were the same as those described in Figure 2 with the exceptions that oxygen concentrations were varied as indicated, the final gas mixtures were made to one atmosphere with argon; and the time of incubation was one hour.

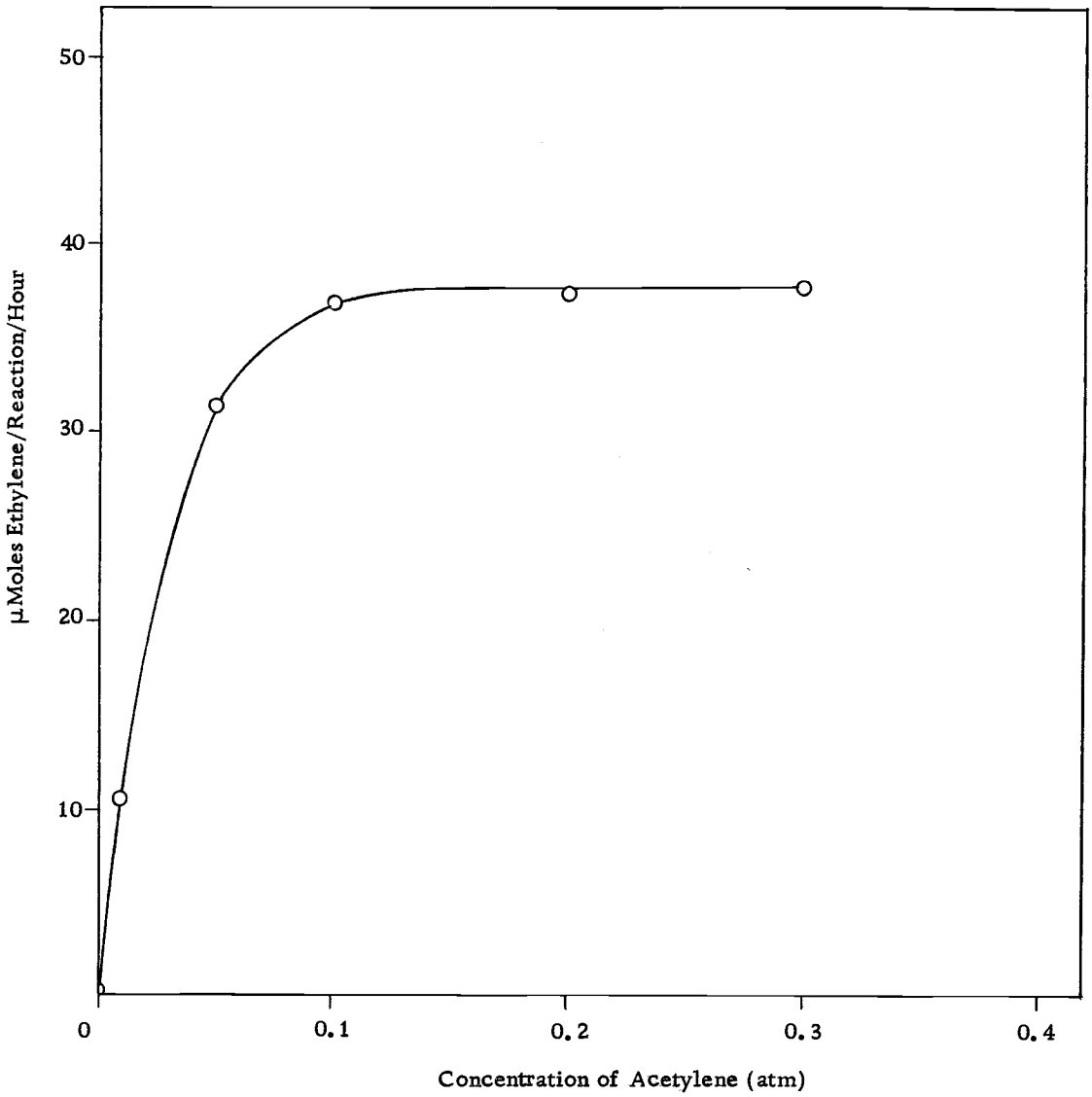

Figure 4. The effect of acetylene concentration on the formation of ethylene by soybean nodules. The conditions were identical with those described in Figure 2 with the exception that acetylene concentrations were varied as indicated, the final gas mixture was made to one atmosphere with argon and the period of incubation was one hour.

reduction. The concentration of oxygen required for the saturation of the acetylene reducing system was considerably less than that reported for the maximum rate of nitrogen reduction in sliced nodules (Burris, 1956), but considerably greater than the value of 0.06 atm which resulted in maximum nitrogen fixation by breis of soybean nodules (Bergersen, 1966).

Data showing the effect of acetylene concentration on the production of ethylene are presented in Figure 4. A concentration of about 0.1 atm of acetylene almost completely saturated the enzyme, and higher concentrations of acetylene did not increase the rate of reduction. From these data an apparent K_m value of 0.022 atm of acetylene was calculated. This value may be compared with the value of 0.025 atm which was reported to be the K_m for nitrogen fixation by slices of soybean nodules (Burris, 1956). The apparent K_m for the reduction of acetylene to ethylene by a purified extract of Clostridium pasteurianum is reported to be 0.03 atm (Hardy and Knight, 1967a).

This investigation has shown that soybean nodules catalyze the reduction of acetylene to ethylene at relatively rapid rates. The conditions necessary for optimum reduction are similar to those reported to be necessary for optimum nitrogen fixation. This suggests that the same enzyme is involved in both nitrogen fixation and acetylene reduction.

Acetylene Reduction by Nodule Breis

The effects of including PVP and ascorbate in the extracting medium on the capacity of the nodule breis to catalyze the reduction of acetylene to ethylene are presented in Table 1. Breis prepared with PVP, but without ascorbate, catalyzed the reduction of acetylene to ethylene at a rate of 7.5 μmoles per hour. When PVP was omitted from the procedure the resulting brei produced only 0.08 μmoles of ethylene per hour. In contrast the nodule brei prepared with PVP and 0.3 M ascorbate in the buffer catalyzed the reduction of acetylene at a rate of over 14 μmoles per hour. The brei prepared in a medium with ascorbate, but without PVP, showed only weak activity. Although all breis were prepared under anaerobic conditions, oxygen was essential in reaction mixtures of acetylene reduction, as has been shown for nitrogen fixation by Bergersen and Turner (1967). In subsequent experiments the optimum concentration of ascorbate in the brei preparation medium proved to be 0.2 M.

A nodule brei was separated by centrifugation into a bacteroid fraction and a soluble protein supernatant containing leghemoglobin. The supernatant solution alone exhibited no capacity to catalyze the reduction of acetylene, but the suspended pellet containing bacteroids and other particulates, rapidly catalyzed the reduction of acetylene. Combination of the supernatant fraction with the bacteroid preparation

Table 1. Acetylene Reduction by Soybean Nodule Brei as Influenced by Polyvinylpolypyrrolidone and Ascorbate in the Brei Preparation.

Preparation of brei	Ethylene produced (μmoles) Incubation time (min) 30	45	60
With PVP	0.97	5.93	7.52
Without PVP	0.04	0.06	0.08
With PVP and 0.3 M Ascorbate	5.34	13.92	14.11
Without PVP, with 0.3 M Ascorbate	0.21	0.46	0.48

Note:

Each reaction bottle (21 ml) contained 10 ml of nodule brei (25 mg protein per ml by microKjeldahl) representing 2.5 gm of 36 day-old nodules. The brei with or without ascorbate and PVP as indicated was prepared in a solution of 20 mM potassium phosphate buffer at pH 7.2 and 1 mM $MgCl_2$. The gas volume (11 ml) in each reaction bottle was composed of 0.25 atm of oxygen, 0.1 atm of acetylene and 0.65 atm of argon. Reaction mixtures were incubated with shaking at 28°C in a water bath. At the times indicated 0.5 ml-samples of gas were removed through the serum cap of each bottle and analyzed for ethylene.

resulted in an increase in the rate of acetylene reduction, but the evidence indicated that the stimulation was due to oxidizable substrates in the supernatant. Since the capacity to catalyze acetylene reduction remained in the bacteroid fraction after washing in buffer to remove leghemoglobin and other components, it was concluded that the enzymes necessary for acetylene reduction were located in the bacteroid preparation, and not in the supernatant.

Acetylene and Nitrogen Reduction by Cell-free Extracts

Requirements for Reduction

A series of experiments were conducted to determine whether cell-free extracts could be prepared from the bacteroid fraction that would catalyze the reduction of acetylene and nitrogen gas. A complete reaction mixture containing cell-free extract and lacking oxygen (Table 2) produced about 9.6 μmoles of ethylene per hour. Reactions lacking ATP, creatine phosphate, $Na_2S_2O_4$ or active extracts produced less than 0.01 μmoles of ethylene per hour. The omission of creatine phosphokinase reduced the rate of the reaction only slightly. In some experiments the reaction was not completely dependent upon ATP. In four successive experiments, however, the reaction was always dependent upon creatine phosphate, $Na_2S_2O_4$, and extract.

Since all cell-free extracts of the bacteroid fraction catalyzed an

Table 2. Requirements for Catalysis of Acetylene Reduction by a Cell-free Extract of a Nodule Bacteroid Preparation.

Reaction conditions	Ethylene Produced (μmoles/hr)
Complete	9.63
Without ATP	< 0.01
Without creatine phosphate	< 0.01
Without creatine phosphokinase	7.27
Without $Na_2S_2O_4$	< 0.01
Complete, but extract boiled	< 0.01

Note:

The complete reaction mixture in a final volume of 6 ml contained: 100 μmoles of potassium phosphate buffer, pH 7.2; 15 μmoles of $MgCl_2$; 6 μmoles of Na_2ATP; 360 μmoles of creatine phosphate; 2.4 mg creatine phosphokinase; 120 μmoles of $Na_2S_2O_4$ and 1.5 ml of cell-free extract containing 61 mg protein per ml. The procedures for gassing, incubation and sampling were those described in Table 1 with the exception that oxygen was omitted and replaced by argon.

ATP and $Na_2S_2O_4$-dependent reduction of acetylene, it was expected that the extracts would also catalyze the reduction of nitrogen gas. Experiments were conducted, therefore, that were similar to the one described in Table 2, with the exception that acetylene was omitted from the reaction, and the gas volume in each tube was made to one atm with nitrogen or argon (Table 3). From the data in Table 3 it is apparent that the complete reaction mixture with an ATP-generating system, $Na_2S_2O_4$, and nitrogen gas produced ammonia at a fairly rapid rate. The omission of the ATP-generating system, $Na_2S_2O_4$, or nitrogen gas resulted in little ammonia production.

In these and other experiments the crude extracts alone contained about 0.2 μmoles of ammonia per mg of protein. Despite the high concentration of endogenous ammonia in the extracts, complete reaction mixtures produced at least 7 μmoles of ammonia during the incubation period. This quantity could be accurately measured in the presence of the endogenous ammonia in the extract.

From the data presented in Table 3 and in experiment I and II of Table 4, it is clear that the production of ammonia or ethylene in the standard assay system was dependent upon nitrogen or acetylene, an ATP-generating system, and $Na_2S_2O_4$. The crude extract, used in experiments in which individual components of the ATP-generating system were omitted (Table 4), had been chromatographed on a Bio-gel P-2 column to remove most of the endogenous ammonia and other low

Table 3. Reduction of Nitrogen Gas by Cell-free Extracts of Soybean Nodule Bacteroid Preparations.

Reaction system	Gas phase	Ammonia in reaction					
		Exp I		Exp II		Exp III	
		Total assayed	Total less NH_3 in Ext[a]	Total assayed	Total less NH_3 in Ext	Total assayed	Total less NH_3 in Ext
		ηmoles/1.5 hr/mg protein					
Complete	N_2	328	120	332	124	402	202
Without ATP system[b]	N_2	227	19	235	27	---	---
Without ATP	N_2	---	---	---	---	406	206
Without $Na_2S_2O_4$	N_2	212	4	220	12	223	23
Without N_2 gas	Ar	---	---	220	12	---	---

[a]The crude cell-free extract contained a relatively high content of endogenous ammonia; therefore, ammonia in extracts after incubation at 28° C during the experiment was determined and subtracted from the total ammonia assayed. In most extracts about 200 ηmoles per mg protein were found. A complete reaction mixture with extract boiled prior to incubation contained 165 ηmoles of ammonia per mg of protein (Exp III).

[b]Creatine phosphate, creatine phosphokinase and ATP.

Note:

The complete reaction mixture in a final volume of 6 ml contained the quantities of reaction components listed in Table 2 with the exception that 150 μmoles of potassium cacodylate buffer at pH 7.2 was used instead of potassium phosphate buffer. A volume of 1.5 ml of bacteroid extract (61 mg/ml, Exp I and II and 41 mg/ml, Exp III) was added to each reaction. Prior to incubation each reaction mixture was evacuated and flushed 3 times with nitrogen or argon as indicated. Samples were assayed for ammonia by the method of Mortenson (1961).

Table 4. Necessary Reaction Components for Cell-free Nitrogen Fixation and Acetylene Reduction.

	Exp. I[a] N_2 fixed (ηmoles/min/ mg protein	Exp. II[a] Ethylene (ηmoles/min/ mg protein	Exp. III[b] $^{15}N_2$ fixed (atom % excess)
Complete[a]	13.8	46.9	3.087
Without N_2, C_2H_4 or $^{15}N_2$	0.0	0.0	.004
Without ATP System[c]	0.1	0.0	.003
Without ATP	0.3	0.1	---
Without creatine phosphate	1.1	4.0	---
Without creatine phosphokinase	0.5	0.9	---
Without $MgCl_2$	0.7	0.4	---
Without $Na_2S_2O_4$	0.0	0.0	.002

[a] In Experiment I and II the complete reaction in 1.5 ml contained: 75 μmoles of tris-Cl buffer at pH 7.5, 10 μmoles of $MgCl_2$, 50 μmoles of creatine phosphate, 7.5 μmoles of Na_2ATP, 0.2 mg of creatine phosphokinase, and 3.8 mg of protein (Bio-gel extract). The gas phase of Experiment I contained 1.0 atm of N_2 while Experiment II contained 0.1 atm of acetylene and 0.9 atm of argon. In both Experiment I and II the incubation period was 20 minutes.

[b] Experiment III was the average of two experiments with the complete reactions in 3 ml containing: 50 μmoles of potassium phosphate buffer at pH 7.0, 7.5 μmoles of $MgCl_2$, 150 μmoles of creatine phosphate, 15 μmoles of Na_2ATP, 0.8 mg of creatine phosphokinase, and 26 mg of protein (crude extract). The gas phase consisted of 0.2 atm of 95% $^{15}N_2$, 0.8 atm of argon and reactions were incubated for one hour.

[c] Na_2ATP, creatine phosphate, and creatine phosphokinase.

molecular weight compounds. The results from experiments represented in Table 4 indicate an absolute requirement for an ATP-generating system with only limited activity when various components of the ATP system were omitted. The slight activity in the absence of added creatine phosphate undoubtedly is due to the 7.5 μmoles of Na_2ATP that was included in the reaction mixture.

Substitution of acetyl phosphate for creatine phosphate, at an equivalent concentration, failed to support nitrogen fixation or acetylene reduction. The possibility that this negative result was caused by a lack of acetokinase in the extract was not investigated.

The omission of $MgCl_2$ in the acetylene reduction and nitrogen fixing experiments (Table 4) resulted in observed rates of one and five percent, respectively of that of the complete reactions. In other experiments, in which the crude extract was not chromatographed on Bio-gel P-2, the reaction rate with $MgCl_2$ omitted was as much as 60 percent of that of the complete reaction. From the results of experiment I and II of Table 4 it is concluded that the experimental conditions necessary for acetylene reduction and nitrogen fixation are similar.

In experiment III of Table 4, the essential components for nitrogen fixation were established in reaction mixtures containing crude extracts. The ^{15}N assay was utilized and thus the effect of endogenous ammonia was not critical. The average of two complete reactions in experiment III (Table 4) produced ammonia with 3.087 atom percent

excess ^{15}N. Omission of the ATP-generating system, $Na_2S_2O_4$, or $^{15}N_2$ resulted in enrichment of ^{15}N in the ammonia from the reactions by extents of 0.004 percent or less.

The evidence that the reduction of nitrogen and acetylene by cell-free extracts of soybean nodules is dependent upon the presence of $Na_2S_2O_4$ and an ATP-generating system is considered conclusive. From these results it is clear that the requirements for catalysis of nitrogen fixation by an extract of nodule bacteroids are similar to those that have been established for extracts of Azotobacter vinelandii (Bulen et al., 1965).

Optimum Conditions for Nitrogen Fixation

A series of experiments were conducted to determine the optimum conditions for catalysis of nitrogen fixation by the bacteroid extract. The data in Figure 5, presented as a modified Lineweaver-Burk plot, show an apparent K_m for nitrogen of 0.052 atm and a V_{max} of 3.47 ηmoles of ammonia per minute per mg of protein. In a series of four experiments the mean K_m for nitrogen was 0.056 ± 0.005 atm. In all these experiments the V_{max} of the reaction was obtained at a pN_2 of about 0.25 atm (e.g. Figure 5). As pointed out by Hardy and Knight (1967), several individuals have observed apparent K_m values for nitrogen in intact organisms that are considerably less than those determined by use of cell-free extracts. Variation in the solubility of

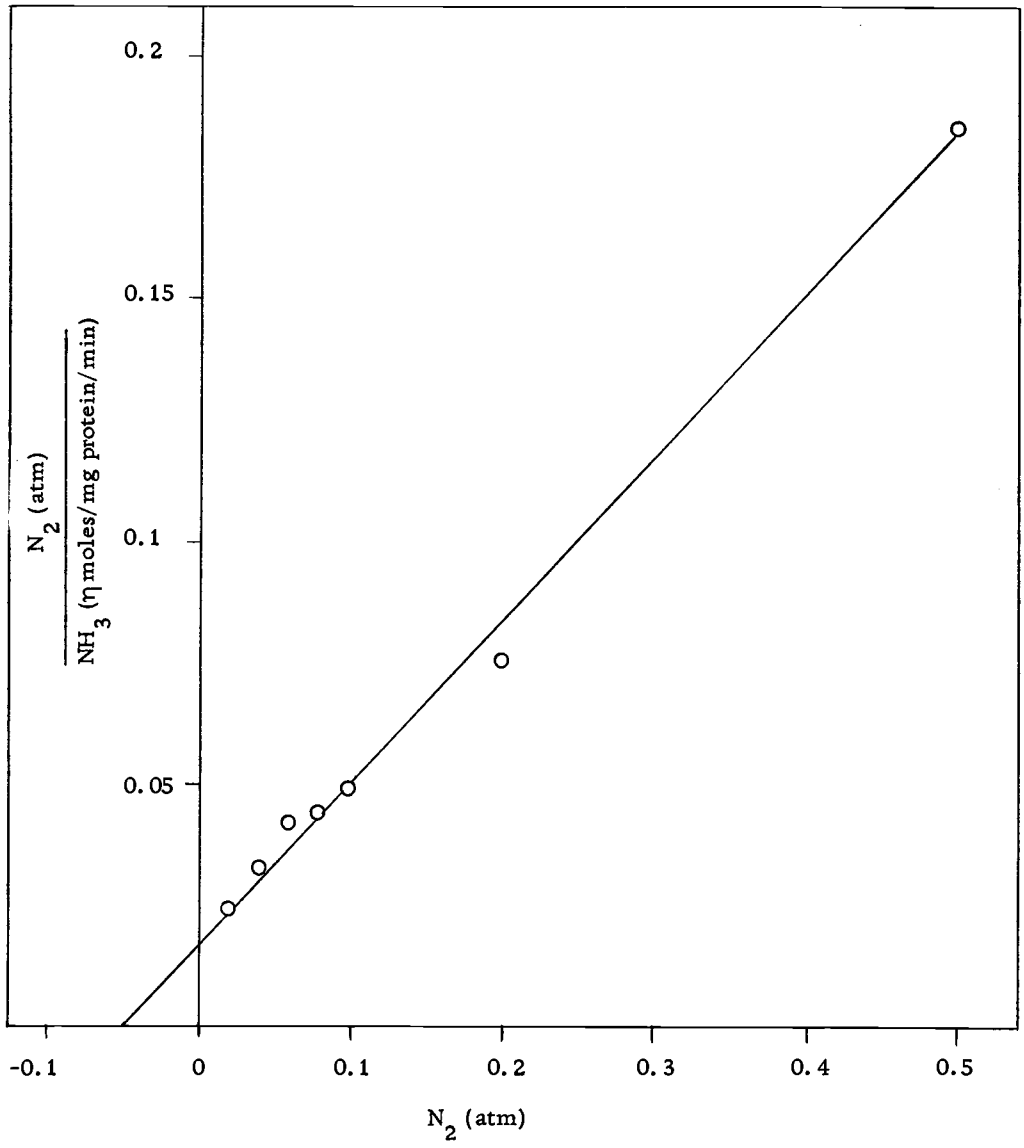

Figure 5. A modified Lineweaver-Burk plot of the rate of N_2 fixation as a function of pN_2 (fixation). Components of reaction mixtures were as described in Experiment III of Table 4 with the exceptions that 14.7 mg of protein (Bio-gel extract) were used and the pN_2 was varied as indicated.

nitrogen in the medium immediately surrounding the nitrogen fixing site was suggested (Hardy et al., 1967) as a possible explanation for the difference.

It is apparent from the data in Figure 6 that the nitrogenase system from soybean-nodule bacteroids was saturated when 10 μmoles (i.e. 3.3 μmoles per ml) of $Na_2S_2O_4$ was included in the reaction mixture. The concentration of $Na_2S_2O_4$ required to achieve one-half V_{max} was about 0.8 μmoles per ml. These values may be compared with 15 and 6 μmoles of $Na_2S_2O_4$ per ml reported necessary for V_{max} and one-half V_{max}, respectively, of nitrogen fixation by cell-free extracts from Azotobacter vinelandii.

No nitrogen reduction was obtained when H_2 (at 0.3 or 0.5 atm) was included in reactions instead of $Na_2S_2O_4$. Experiments in which pyruvate, α-ketoglutarate, β-hydroxybutyrate, NADH, or NADPH were utilized as possible electron donors instead of $Na_2S_2O_4$ produced negative results.

Approximately 135 μmoles of creatine phosphate (45 μmoles per ml) was sufficient for saturation of the nitrogenase system from nodule bacteroids (Figure 7). According to Bulen et al. (1965), the cell-free system from Azotobacter vinelandii required approximately 60 μmoles of creatine phosphate per ml to obtain maximum velocity. The reaction was not completely dependent upon creatine phosphate (Figure 7). This low activity would be expected because 15 μmoles of ATP was included

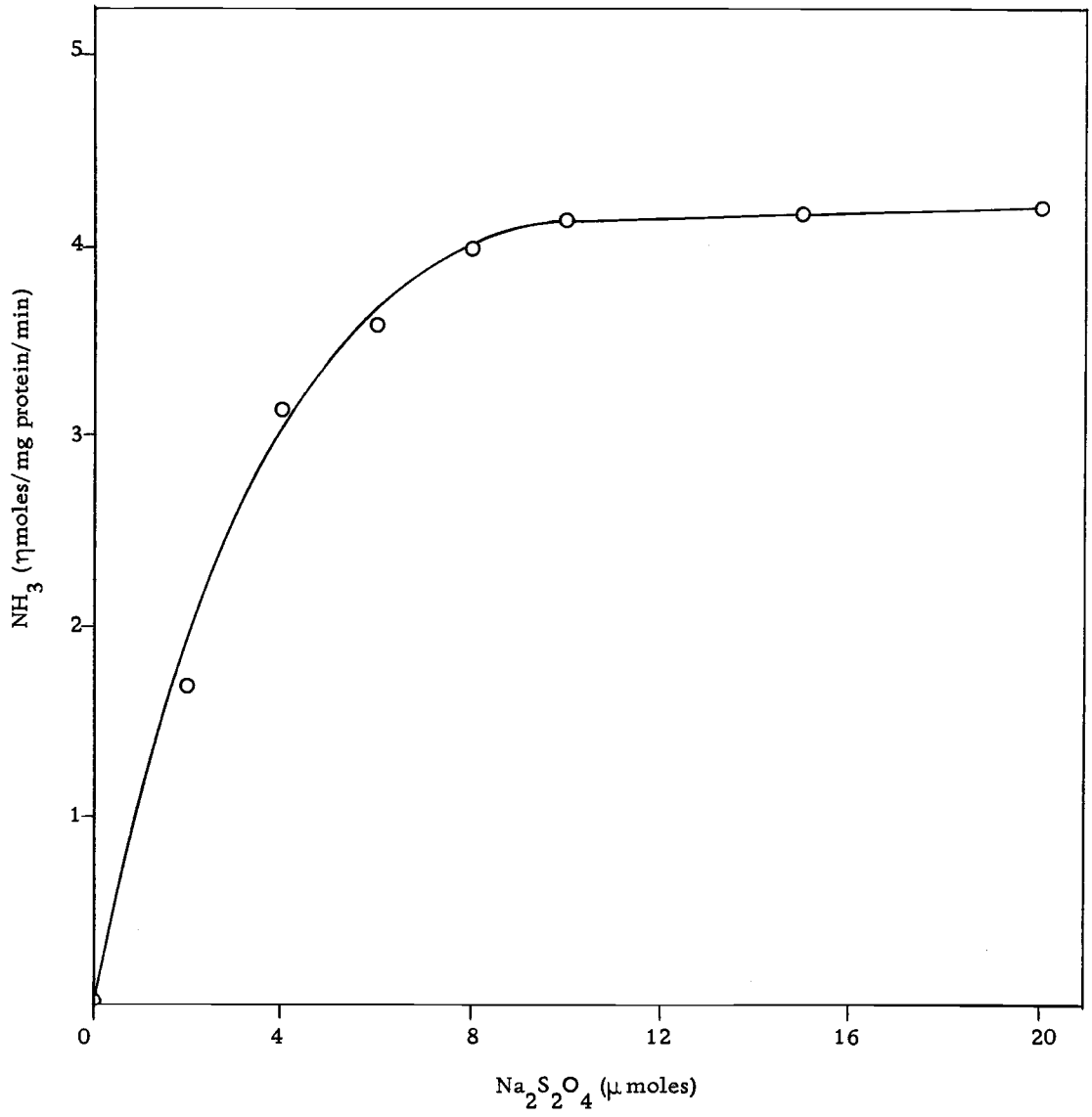

Figure 6. The effect of the concentration of $Na_2S_2O_4$ on the rate of nitrogen fixation. Each reaction mixture (3 ml) contained 13.8 mg of protein (Bio-gel extract) and components listed in the complete reaction mixture of Experiment III, Table 4 with the exception that $Na_2S_2O_4$ was varied as indicated.

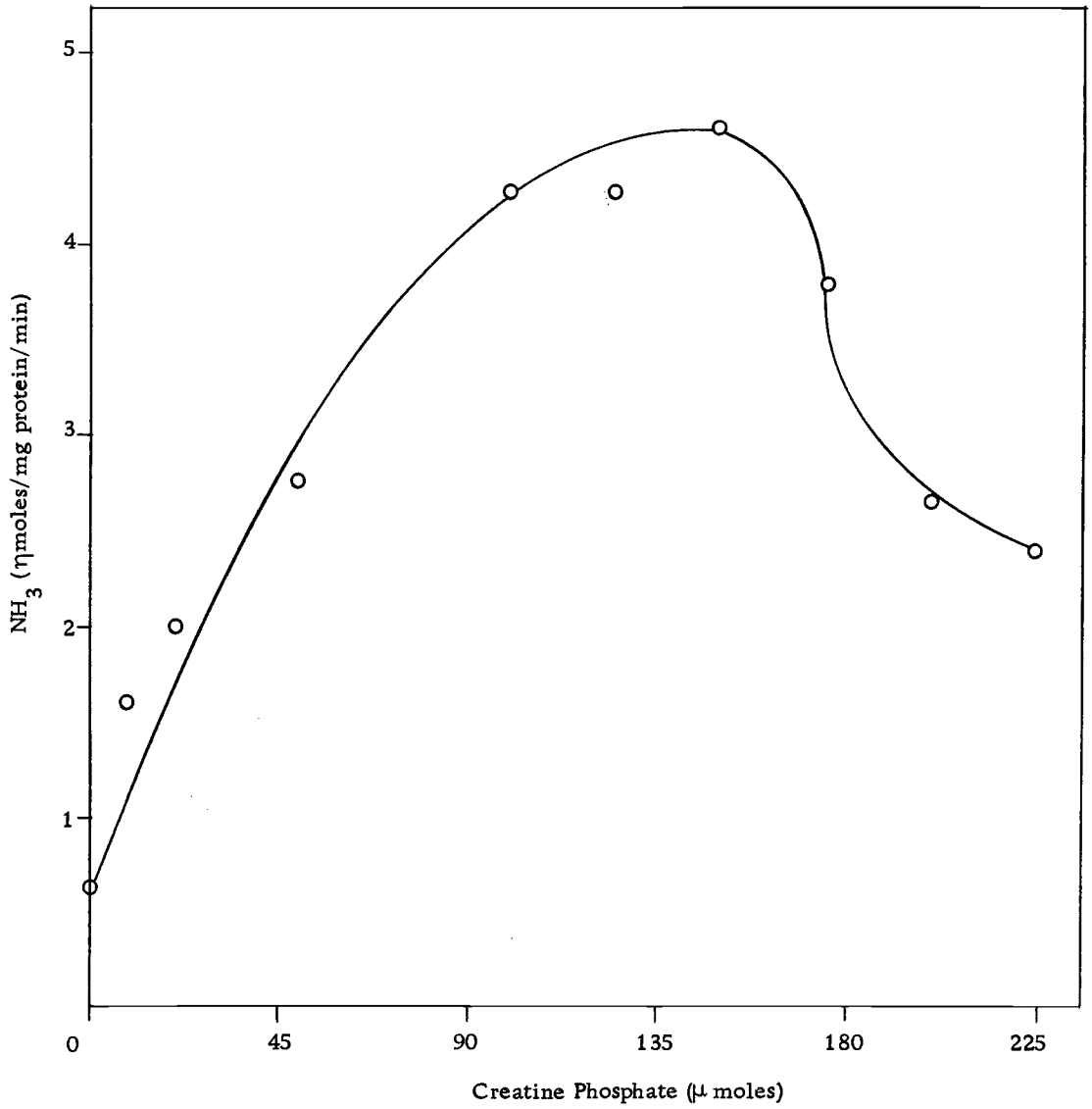

Figure 7. The rate of N_2-fixation as a function of creatine phosphate concentration. Each reaction (3 ml) contained 15.5 mg of protein (Bio-gel extract) and other components as listed in the complete reaction of Experiment III, Table 4, with the exception that creatine phosphate was varied as indicated.

in each reaction.

After establishing the optimum concentration of substrates and cofactors for nitrogen fixation by the bacteroid system the relationship between the reaction rate and the enzyme concentration was determined. The data in Figure 8 show that the rate of ammonia synthesis was directly proportional to the amount of enzyme in the reaction throughout the range of 0 to 21 mg protein. In most experiments about 15 mg of protein was utilized in each 3 ml reaction mixture.

The data presented in Figure 9 reveal that the rate of nitrogen fixation by the cell-free extract was linear during the first 60 minutes of the incubation period, but decreased appreciably during the second hour of incubation.

Gas Evolution

Hydrogen evolution is reported to be associated with nitrogen fixation by extracts of Azotobacter vinelandii (Bulen et al., 1965, and Burns and Bulen, 1965), by soybean nodules (Hoch, Little and Burris, 1957), and by nodule breis (Bergersen, 1966b and 1967). Experiments were conducted, therefore, to determine the quantity of hydrogen gas evolved in reactions containing bacteroid extracts. As illustrated in Figure 10 a reaction carried out under an atmosphere of nitrogen evolved 24 μl of gas in 50 minutes. An average of 3.0 μmoles of ammonia were produced under these conditions. When the reaction was

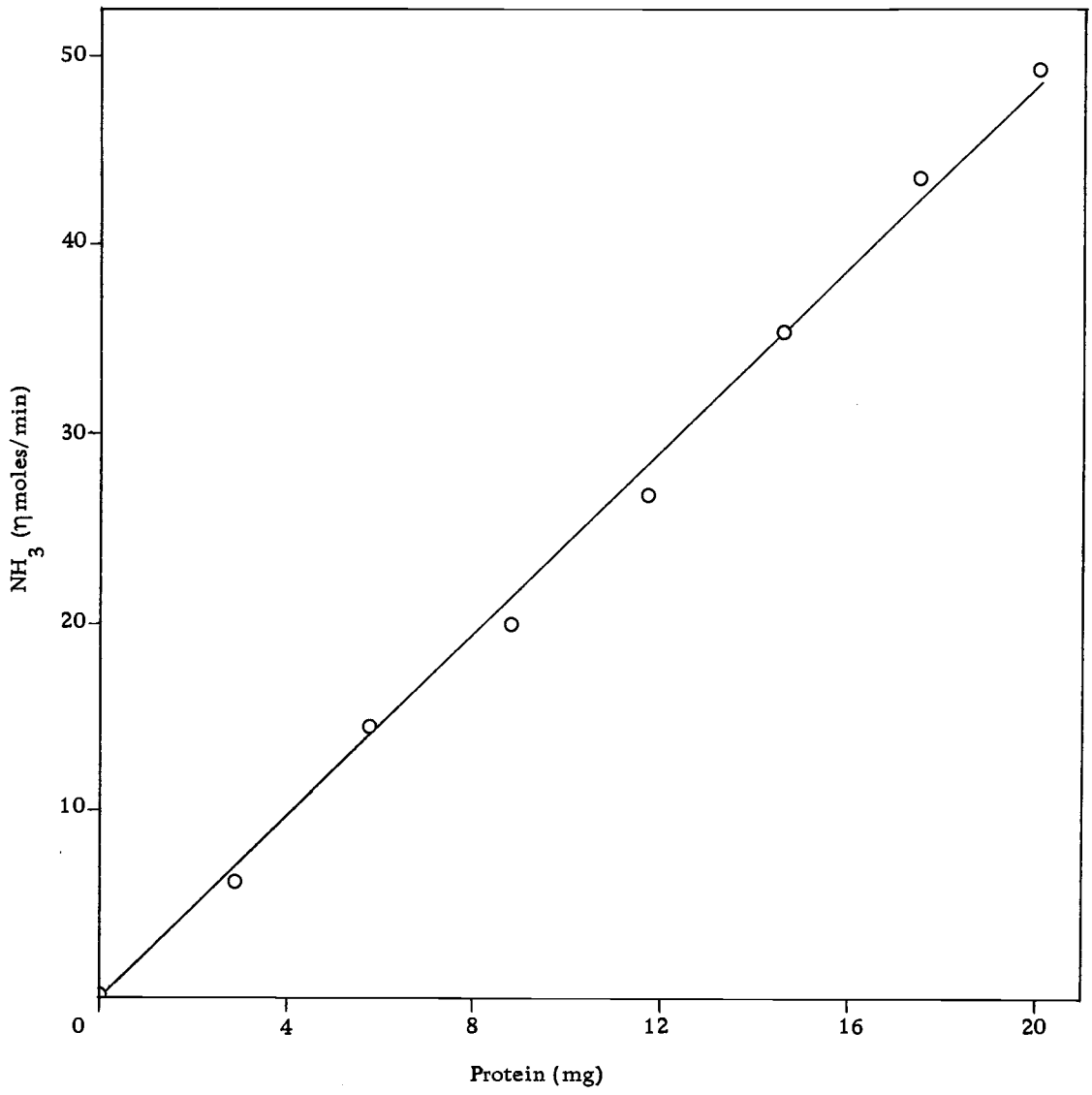

Figure 8. The relationship of the rate of nitrogen fixation to quantity of enzyme per reaction. The conditions were as described in the complete reaction of Experiment III, Table 4 with the exception that the quantity of Bio-gel extract of bacteroids was varied as indicated.

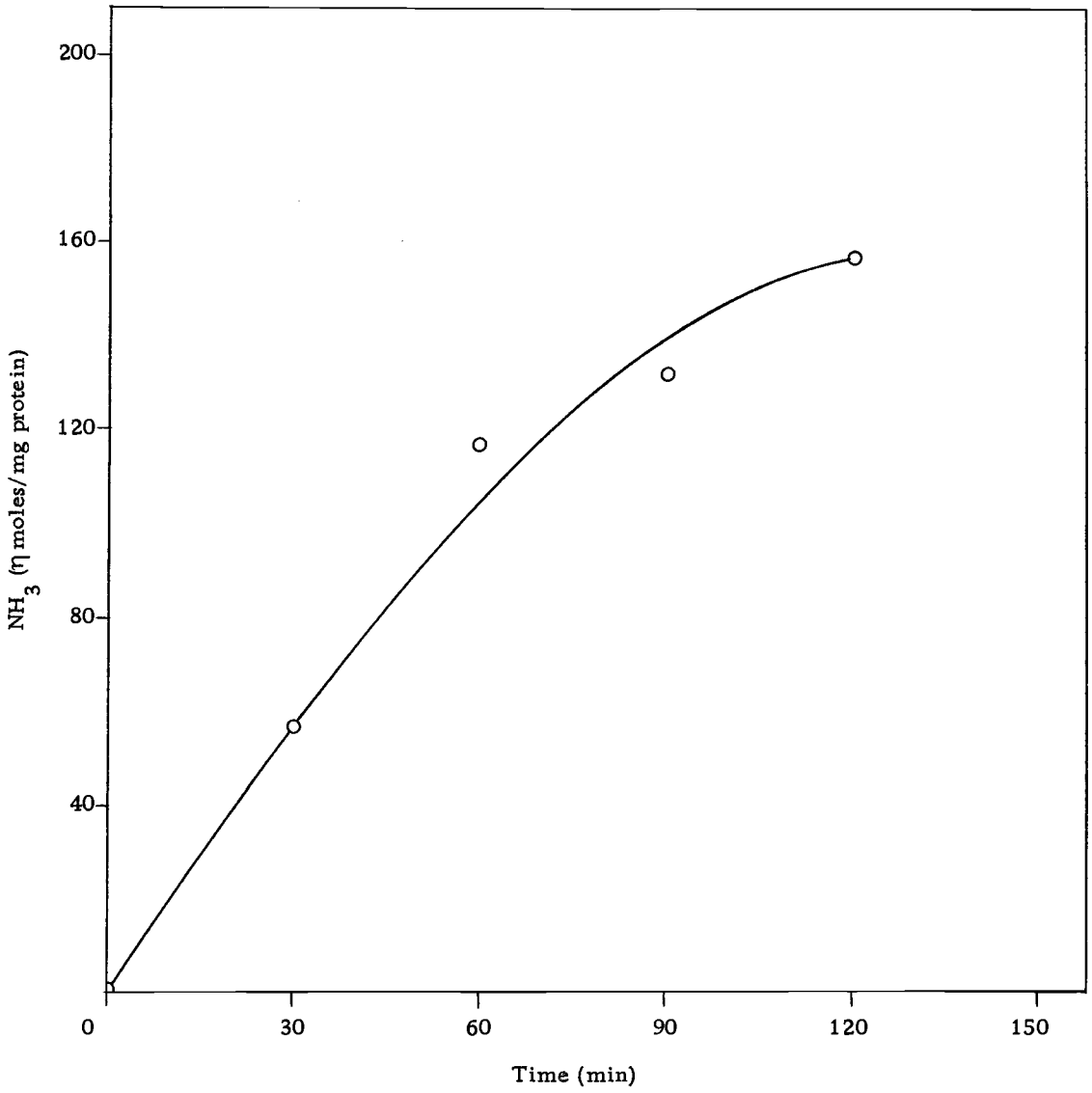

Figure 9. Time-course of the nitrogen fixation reaction. Each reaction contained 15 mg of protein (Bio-gel extract) and the quantities of components listed for the complete reaction mixture of Experiment III, Table 4. The period of incubation varied as indicated.

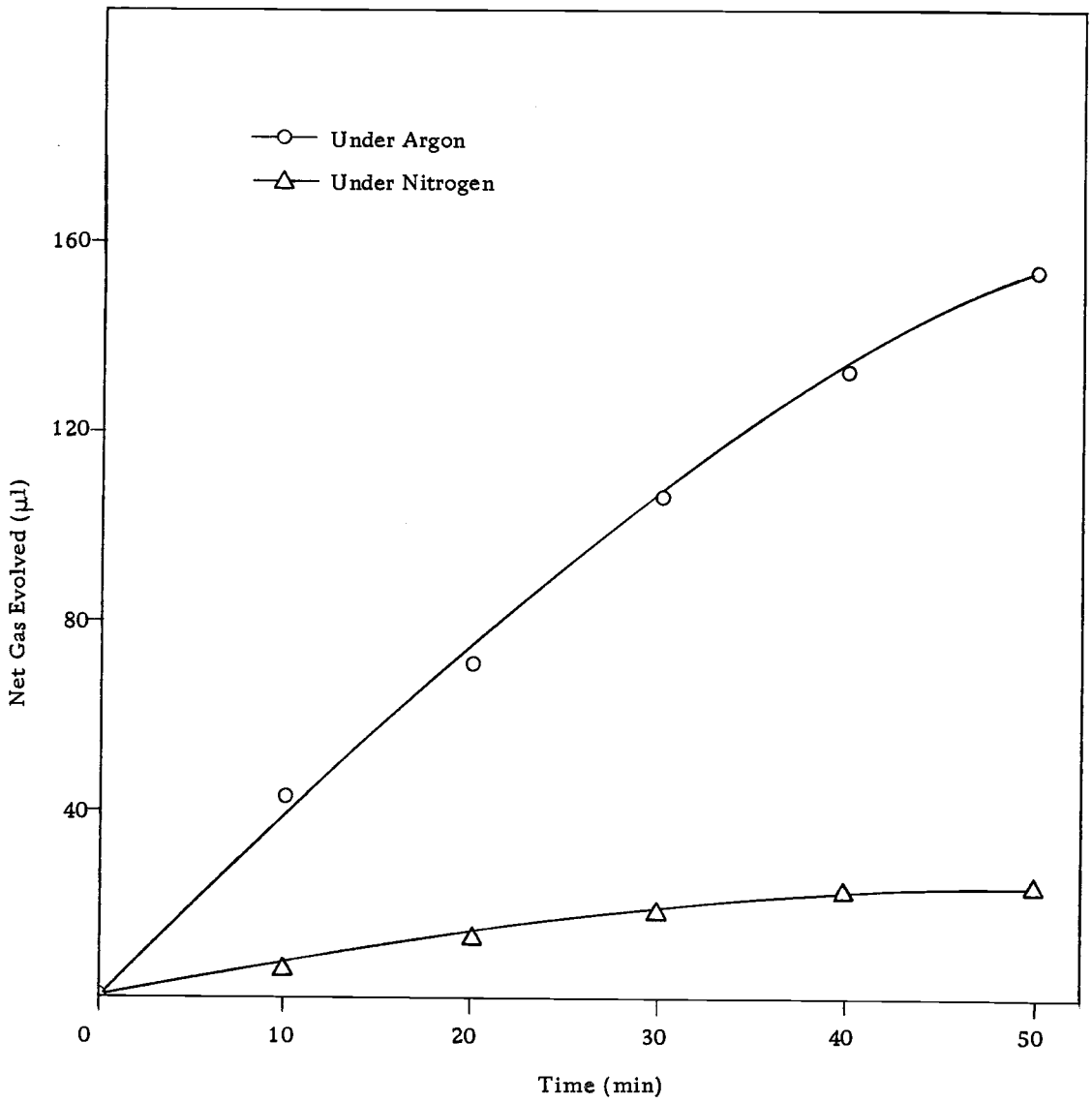

Figure 10. The enzyme dependent evolution of gas under nitrogen and argon. Each reaction mixture of 3.0 ml in a Warburg flask contained 12 mg of protein (Bio-gel extract) and the components of the complete reaction mixture listed in Experiment III, Table 4, with the exception that the gas phase varied as indicated. The evolution of gas in the presence of extract was dependent upon the ATP system and $Na_2S_2O_4$. For further details of the manometric procedure see Materials and Methods.

carried out under an atmosphere of argon, 152 μl of gas was evolved. The evolution of gas under argon or nitrogen was dependent upon the addition of an ATP-generating system and $Na_2S_2O_4$.

The gas evolved was identified as hydrogen in parallel experiments. In these tests a small piece of tape covered with a mixture of a palladium and charcoal powder was placed in the sidearm of each flask. At the termination of the experiment 200 μmoles of a solution of $K_4Fe(CN)_6$ was injected into each flask. The tenfold excess of $K_4Fe(CN)_6$, in respect to amount of $Na_2S_2O_4$ added, provided assurance that all residual $Na_2S_2O_4$ was oxidized. After equilibration for five minutes 100 μl of oxygen was added to each reaction and the gas uptake was determined immediately. The decrease in the volume of gas was consistent with a reaction of hydrogen and oxygen. It was concluded, therefore, that the ATP-dependent evolution of gas under nitrogen and argon was due to the production of hydrogen.

The synthesis of 3 μmoles of ammonia in the reaction (Figure 10) would require 9 μeq of reductant, and the evolution of hydrogen gas under nitrogen would require a further 2.2 μeq of reductant, therefore a total of 11.2 μeq of reductant would be needed for the reaction under nitrogen. The reaction carried out under argon produced 152 μl of hydrogen. This quantity of gas is equivalent to 13.6 μeq of reductant. From these data it is apparent that the quantities of reductant accounted for in reactions under nitrogen and argon are in fair

agreement, and indicate a constant consumption of reductant regardless of the function of the nitrogenase system. These results in general are consistent with those that have been obtained in similar experiments in which extracts of Azotobacter vinelandii (Bulen et al., 1965 and Burns et al., 1965) and Clostridium pasteuranium (Hardy, Knight and D'Eustachio, 1965) function both in nitrogen fixation and ATP-dependent hydrogen evolution, and leads to the hypothesis that both activities are catalyzed by the nitrogenase system.

Inhibition by Hydrogen

Experiments with red clover (Wilson, Umbreit and Lee, 1937; Wilson and Umbreit, 1938), in which different mixtures of hydrogen and nitrogen were utilized to investigate the effect of pN_2 on the fixation of nitrogen, lead to the discovery that hydrogen is a specific inhibitor of nitrogen fixation. As pointed out in a recent review (Hardy et al., 1967), hydrogen inhibits nitrogen fixation by a variety of nitrogen fixing organisms. In all but a few cases the inhibition has been shown to be of the competitive type. For some unexplained reason high concentrations of phosphate (0.05 M) in reaction mixtures, using nitrogenase from Clostridium pasteurianum, resulted in uncompetitive inhibition by hydrogen while low concentrations of phosphate (0.006 M) exhibited competitive inhibition between hydrogen and nitrogen (Lochshin and Burris, 1965).

From the data presented in Figures 11 and 12 it is clear that hydrogen inhibits the catalysis of nitrogen fixation by the bacteroid extract in a low concentration of phosphate buffer. At a pN_2 of 0.25 atm where maximum nitrogen fixation is obtained, the addition of 0.25 atm of hydrogen results in 67 percent inhibition of the rate of nitrogen fixation (Figure 11). In a similar experiment (Figure 12) in which 0.05 atm of hydrogen was added, nitrogen fixation at 0.25 atm of nitrogen was inhibited 29 percent. The data from Figure 11 were plotted by the method of Lineweaver and Burk, and it was concluded that the inhibition of nitrogen fixation by hydrogen was primarily competitive, and that the K_i for hydrogen was 0.058 atm. In the experiment (Figure 12) in which reactions contained 0.05 atm of hydrogen, a K_p of 0.082 was obtained. From this value, and a K_m for nitrogen of 0.055 atm, a K_i for hydrogen of 0.106 was calculated. It is clear that these values are considerably smaller than 0.32 atm which was the value reported by Dilworth et al. (1965) to be the K_i for hydrogen for the cell-free system from Clostridium pasteurianum. Re-evaluation of both K_m and inhibitor constants with a more highly purified enzyme preparation from nodule bacteroids may lead to different values.

Purification of the Nitrogenase System

A partial purification of nitrogenase was achieved using the following procedures. Crude extract in 0.05 M TES buffer at pH 7.4 was

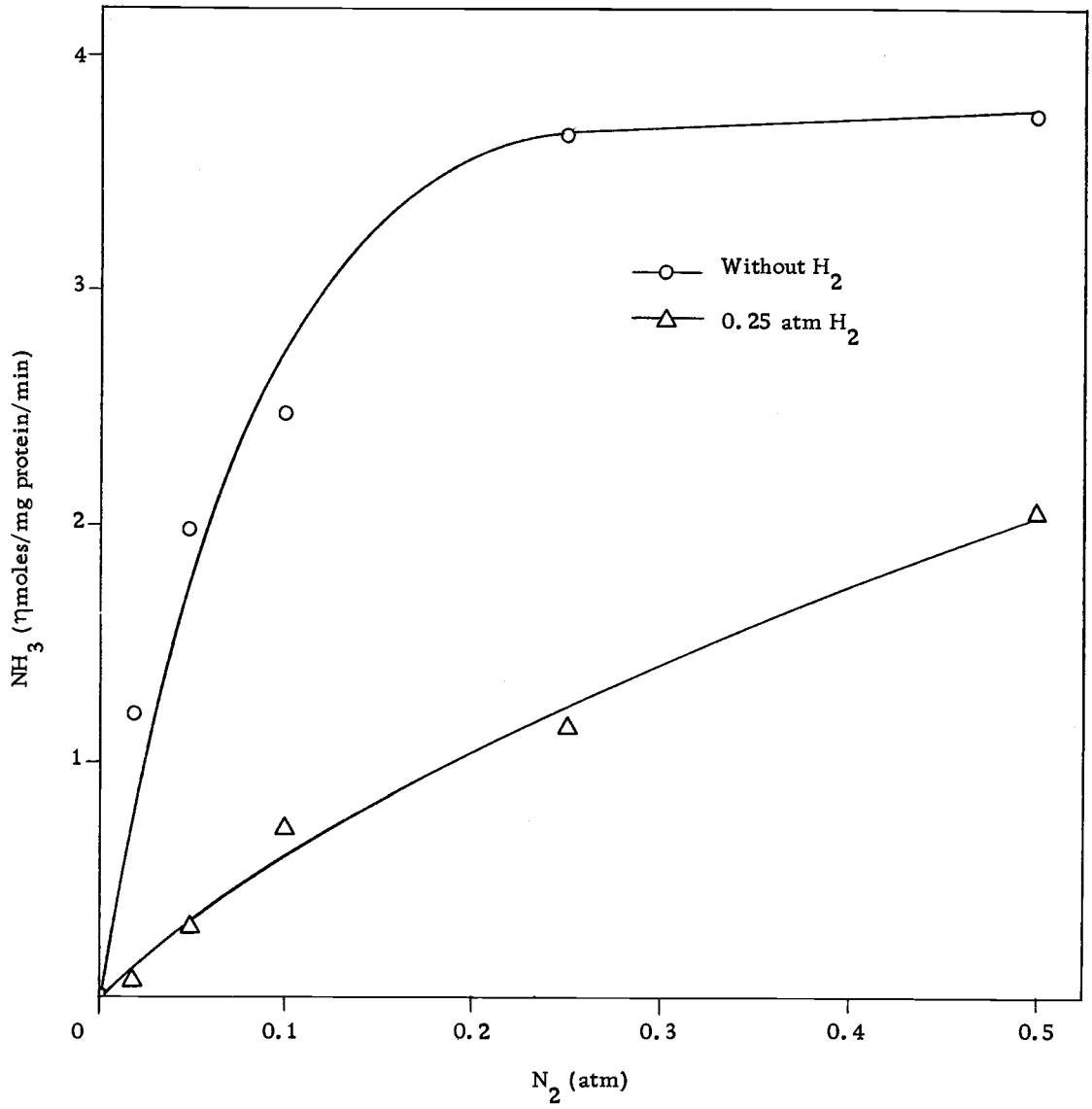

Figure 11. Inhibition of nitrogen fixation by hydrogen. The conditions were as described for Experiment III, Table 4, with exceptions that 15 mg of protein (Bio-gel extract) were utilized, the gas phase was varied as indicated and each reaction contained 18 μmoles phosphate and 60 μmoles of cacodylate buffer pH 7.0.

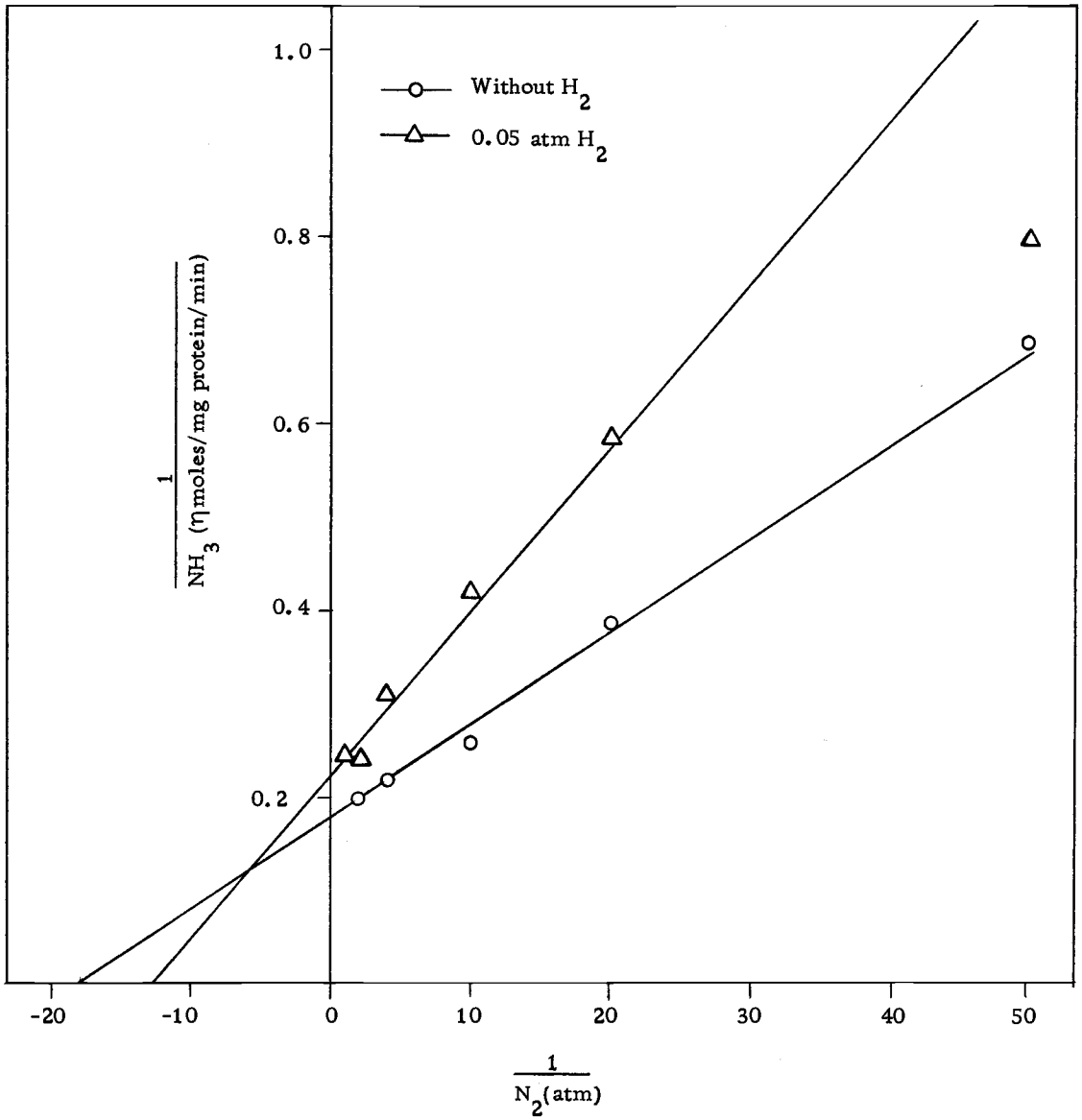

Figure 12. A Lineweaver-Burk plot illustrating the inhibition of nitrogen fixation by hydrogen. The conditions were as described in Experiment III of Table 4 with the exceptions that the gas phase was varied as indicated, and each reaction in 3 ml contained 16 mg of protein (Bio-gel extract), 18 μmoles of phosphate and 60 μmoles of cacodylate buffer at pH 7.0. Lines were located by the method of least squares. The point where 0.05 atm of hydrogen and 0.02 atm of nitrogen ($1/N_2 = 50$) was utilized and not included in the calculation. Values for $-1/Kp$ are -12.3 and -18.2, respectively.

treated with protamine sulfate, heated at 55°C for 3 min, and centrifuged. The supernatant was applied to a column of DEAE-cellulose (Whatman DE32) equilibrated with 0.05 M TES buffer at pH 7.4. Considerable protein material failed to adhere to the column and was eluted rapidly. The column was eluted with increasing concentrations of $MgCl_2$ in 0.5 M TES buffer at pH 7.4 (Figure 13). The addition of 0.025 M $MgCl_2$ to the buffer eluted a fraction (between 100 and 110 ml) that contained a relatively high iron content. A major peak of protein, green-to-brown in appearance, was removed from the column between 170 and 190 ml of eluate. This fraction contained 95 percent of the nitrogenase in the eluate and relatively high concentrations of iron and molybdenum. The application of 0.2 M $MgCl_2$ in 0.05 M TES buffer to the column resulted in the elution of additional proteins that were also relatively high in iron content. The results summarized in experiment I of Table 5 show that the nitrogenase enzyme was eluted as one fraction. Assays of various combinations of fractions for nitrogenase activity provided no evidence of separation of the enzyme into more than one component.

The major colored fraction from the experiment (170 to 190 ml of eluate, Figure 13) was centrifuged for 16 minutes at 52,000 rpm in a Spinco Model E centrifuge. The Schlieren patterns indicated that approximately 88 percent of the protein in this fraction could be accounted for by the major colored component. After centrifugation

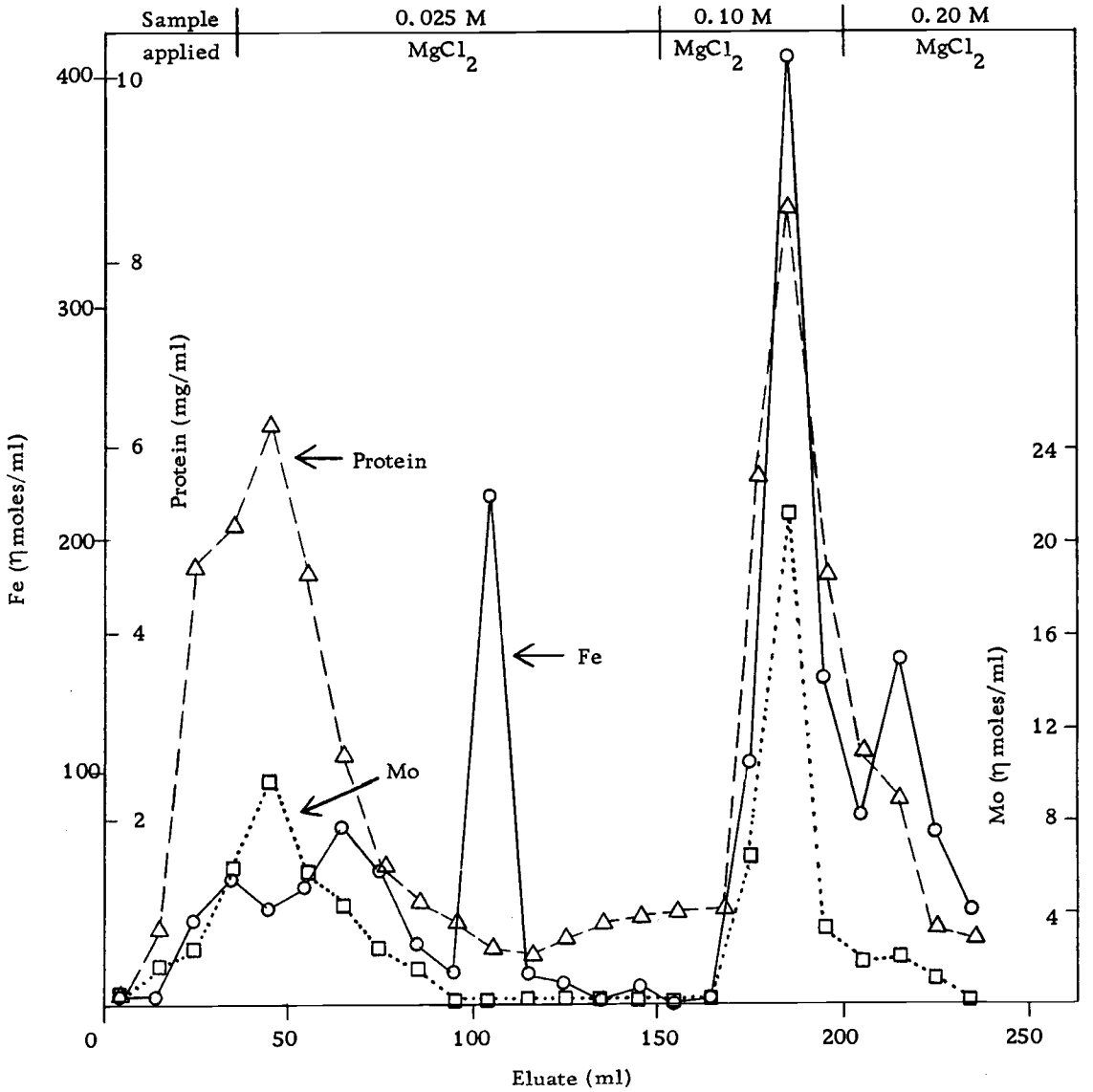

Figure 13. Chromatography of soybean bacteroid nitrogenase in TES buffer. In preparation for the chromatography, 40 ml of crude bacteroid extract (680 mg of protein) in 0.05 M TES buffer at pH 8.0 was treated with 50 mg of protamine sulfate per gm of protein. The extract was placed in a centrifuge tube, sealed under argon and heated for five minutes at 55°C, cooled to 5°C and then centrifuged at 35,000 x g for 30 minutes. The supernatant was transferred to an anaerobic DEAE-column (2.5 x 7 cm) that previously had been equilibrated with 0.05 M TES at pH 7.4. Fractions of 10 ml were collected and assayed for protein, nitrogenase activity, iron and molybdenum.

Table 5. A Summary of the Partial Purification of Bacteroid Nitrogenase.

	Experiment I		Experiment II	
	Specific Activity	Activity Recovery	Specific Activity	Activity Recovery
	ηmoles N_2 fixed/ min/mg protein	%	ηmoles N_2 fixed/ min/mg protein	%
Crude extract	8.1	100	9.2	100
Heat and protamine sulfate	13.4	81	---	---
Protamine sulfate	---	---	16.0	85
Fraction 1	---	---	0.0	0
Fraction 2	---	---	5.6	2
Fraction 1 and 2	17.2	24	72.5[a]	29

[a]This value is based upon the protein content of fraction 2 which was limiting. In this reaction 0.2 ml of each fraction (2.1 mg of protein from fraction 1 and 0.68 mg from fraction 2) was utilized in the assay.

Note:

The reaction mixtures in 1.5 ml contained the components described in Table 4 experiment I and II with the exception of protein concentration.

for 12 minutes, the major peak in the Schlieren pattern separated into two components with apparent S values of 8.0 and 4.5. Although further purification is essential, these results provide tentative evidence that the two major components observed in Schlieren patterns may represent two components of the bacteroid nitrogenase.

In collaboration with Dr. Robert Klucas another fractionation procedure was utilized that was essentially that employed by Kelley et al. (1967) for the resolution of the nitrogenase from Azotobacter vinelandii into two fractions. The major differences between this procedure and that described in Figure 13 are as follows: the extract was prepared in 0.025 M tris-Cl at pH 7.4, the column was equilibrated with tris rather than TES buffer, and a NaCl solution was used for elution prior to the addition of $MgCl_2$. The DEAE column was first eluted with 0.15 M NaCl in 0.025 M tris-Cl at pH 7.4, which resulted in the elution of considerable protein (Figure 14). The addition of 0.035 M $MgCl_2$ removed a major greenish-brown component between 160 and 170 ml of eluate. This fraction contained considerable iron and molybdenum and is referred to in Figure 14 as fraction one. The addition of 0.06 M $MgCl_2$ followed by 0.1 M $MgCl_2$ resulted in the eluting of other colored fractions containing appreciable iron, but only the 255 to 265 ml eluate (fraction 2, Figure 14) showed activity when combined with fraction 1. The final protein collected from the column between 180 and 330 ml of eluate was blue-gray in appearance and did not possess any nitrogenase

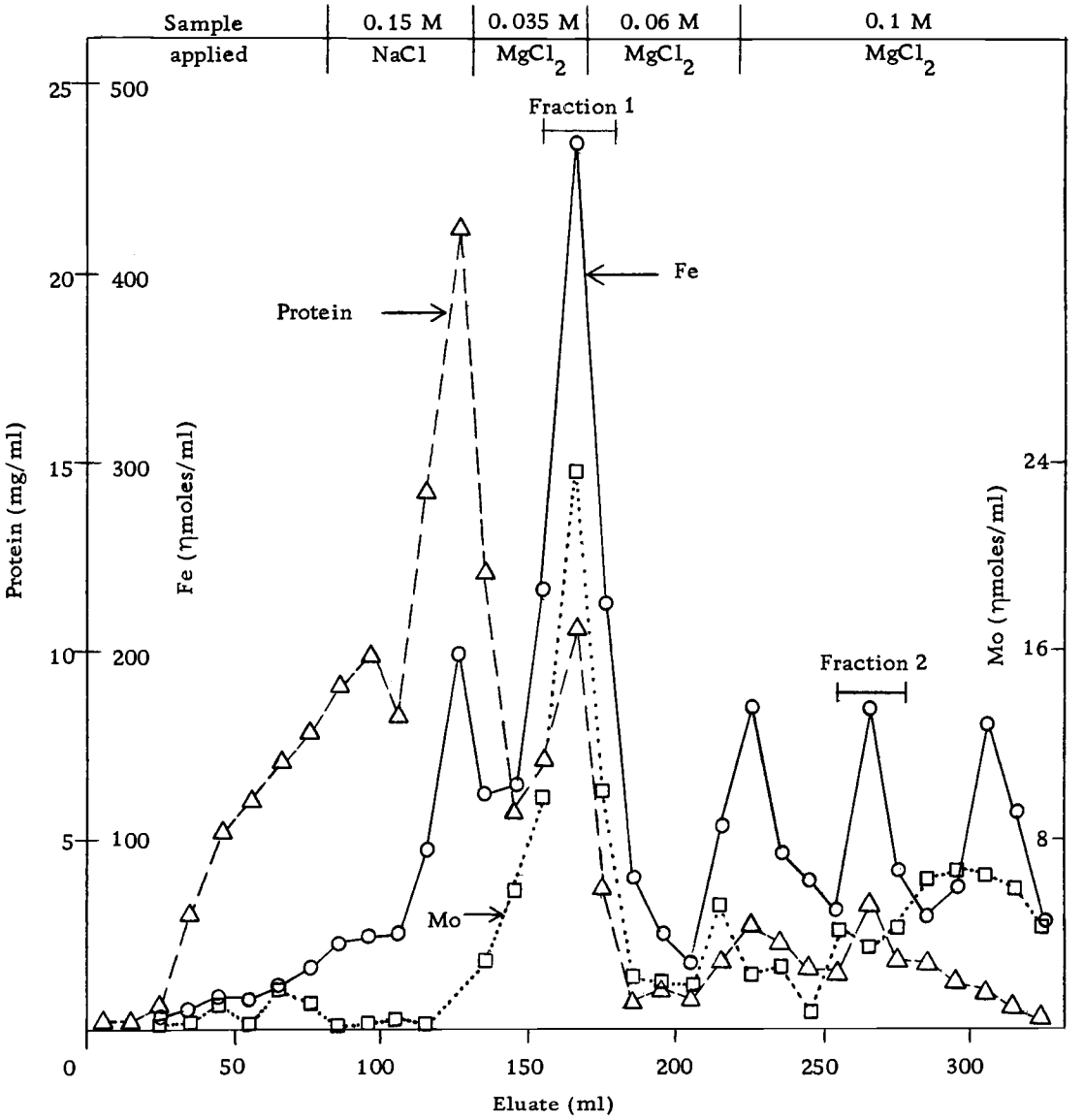

Figure 14. Fractionation of bacteroid nitrogenase into two components. In the preparation for chromatography, 74 ml of crude extract (950 mg protein in 0.025 M tris-Cl pH 7.4) was treated with protamine sulfate (50 mg/g of protein) and centrifuged at 35,000 x g for 10 min. The supernatant liquid was placed on an anaerobic DEAE-cellulose column (2.5 x 13 cm) that previously was equilibrated with 0.025 M tris-Cl at pH 7.5. The column was eluted with salt solutions, all prepared in 0.025 M tris-Cl at pH 7.4, as shown in the diagram. Fractions of 10 ml were collected and assayed for nitrogenase activity, protein, iron and molybdenum.

activity.

The results summarized in experiment II of Table 5 show that fraction 1 exhibited no nitrogenase activity when assayed alone. Weak activity was shown by fraction 2 alone, but a combination of equal volumes of fraction 1 and 2 resulted in a striking increase in specific activity. From these results it appears that the nodule bacteroid nitrogenase consists of at least two protein components. Investigation of the homogeneity of the two fractions in the Spinco Model E centrifuge has been complicated by precipitation of the fractions during the operation. Gel electrophoresis revealed a major component and several minor components in each of the two fractions, but air was not excluded during the procedure, and thus the extent of denaturation during electrophoresis is not known. From iron and molybdenum analysis and from spectra showing fairly broad absorption bands in the region near 415 mμ, these fractions appear similar to the iron-molybdenum protein and the iron protein identified as components of the nitrogenase from Azotobacter vinelandii (Bulen et al., 1966) and Clostridium pasteurianum (Mortenson et al., 1967).

A third fractionation was employed that was similar to the one described in Figure 14, with the exception that the protamine sulfate precipitation step was omitted, and a preliminary polypropylene glycol (PPG) precipitation was carried out. Sufficient PPG (P-400) was added to the extract while stirring in an ice bath to give a final concentration

of 20 percent PPG. The extract was centrifuged at 35,000 x g for 15 minutes and the supernatant was treated, as indicated, with additional PPG to result in a 35 percent PPG solution. This extract was centrifuged at 15,000 x g for 15 minutes, the supernatant decanted, and the precipitated protein dissolved in 0.05 M tris-Cl buffer at pH 7.5. Chromatography of the PPG fraction on DEAE-cellulose separated the nitrogenase system into two components, neither of which exhibited activity alone. An experiment was performed (Figure 15) where the concentration of fraction 1 was held constant at 1.5 mg of protein per reaction, and fraction 2 was varied up to a maximum of 2.7 mg of protein. In another experiment (Figure 15) fraction 2 was held constant at 0.9 mg of protein per reaction and increasing increments of fraction 1 were added. Increasing the concentration of the varied fractions yielded typical enzyme saturation curves. The results presented in Figure 15 indicate that it is essential, when determining the specific activity of a fraction, to provide an excess of the other fraction, and to establish the specific activity of the fraction by varying it in the range where the response approaches linearity. Optimum combinations of the two fractions can be obtained by observing maximum specific activity from combinations based on total protein. Such data, however, are of little value for the assessment of purification of individual fractions.

The capacity of soybean nodules and nodule extracts to carry out nitrogenase-dependent acetylene reduction has been used as a sensitive

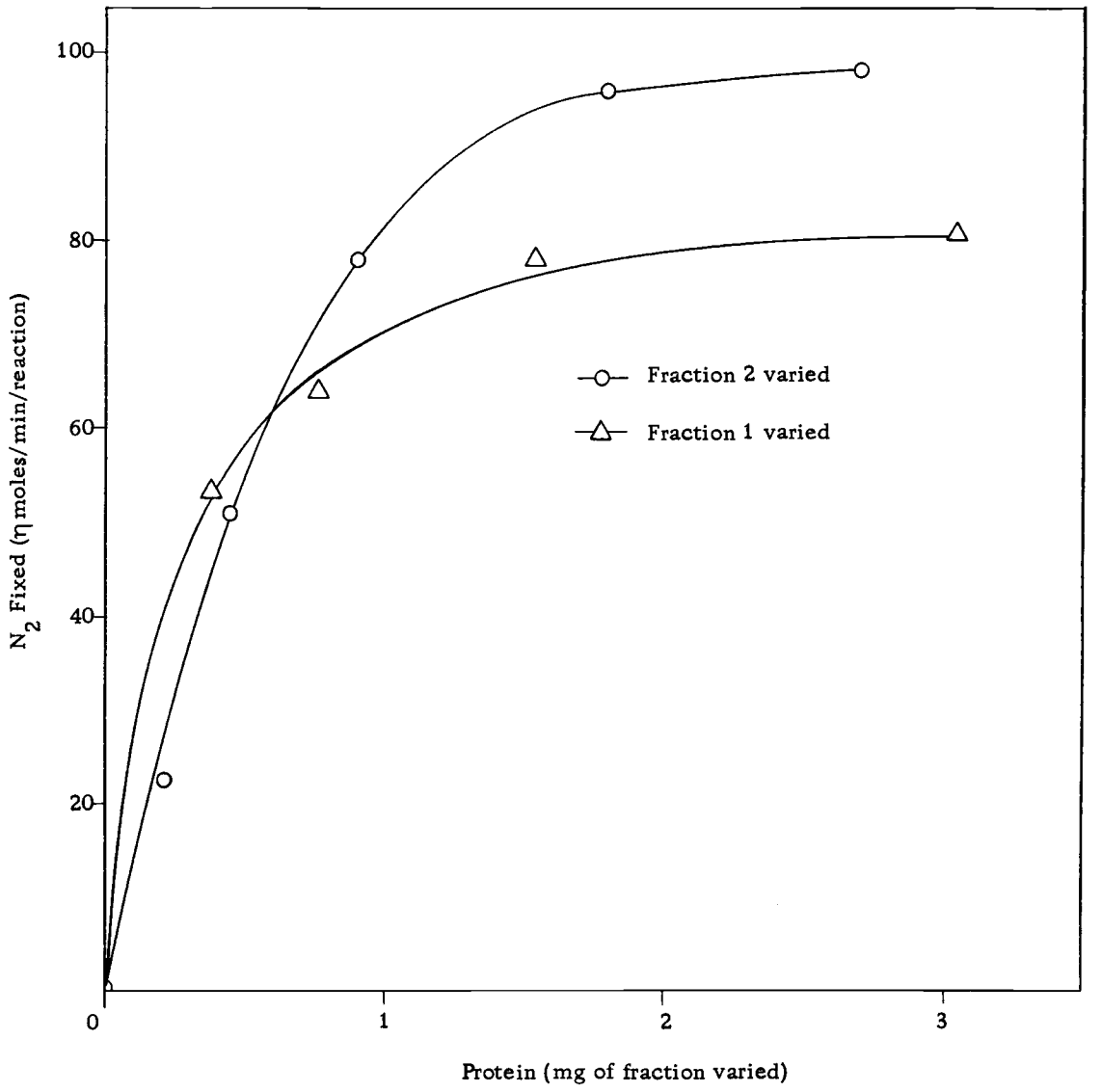

Figure 15. Nitrogenase activity as influenced by different combinations of fractions. The fractionation procedure was as described in the text. Each reaction in the experiment where fraction 2 was varied contained a constant amount of fraction 1 (1.5 mg protein) and the concentrations of fraction 2 indicated. In the experiment where fraction 1 was varied a constant amount of fraction 2 (0.9 mg protein) was added and fraction 1 was varied as indicated.

assay leading to the initial demonstration of cell-free nitrogen fixation in extracts of nodule bacteroids. Since the reduction of acetylene to ethylene requires two electrons per molecule, whereas six electrons are required for the reduction of nitrogen to two molecules of ammonia, it is of considerable interest to investigate the relationship between acetylene reduction and nitrogen reduction by crude extracts and by partially purified fractions.

As indicated in Table 6, crude extract, a polypropylene glycol precipitate, and a combination of fraction 1 and 2 all rapidly catalyze the reduction of both acetylene and nitrogen. Fraction 1 alone exhibited no activity in either assay, but fraction 2 alone exhibited considerable activity in both assays and therefore must have been contaminated with fraction 1. An average of ratios of rates of acetylene reduction to rates of nitrogen reduction gives a value of 3.19, which is close to the theoretical value of three. With the exception of the ratio obtained with fraction 2, ratios of rates of acetylene reduction to nitrogen reduction were very close to three. The absolute amount of nitrogen reduced was very low in the test where fraction 2 was assayed alone, and thus considerable error might have been associated with this measurement. These data support the conclusion that acetylene reduction and nitrogen reduction are catalyzed by the same enzyme. Apparently the flow of electrons to the substrate is limiting the rate of the reaction rather than the interaction of the substrate with the active site (Hardy et al., 1968).

Table 6. Relationship Between the Reduction of Acetylene and Nitrogen by Extracts and Fractions of Nodule Bacteroids.

Type of Extract	Rate of reduction (ηmoles/min/mg protein) C_2H_2	N_2	Ratio of reduction C_2H_2:N_2
Crude	35.2	11.8	2.98:1
20-35% PPG ppt.	76.1	27.4	2.78:1
Fraction 1	0.0	0.0	
Fraction 2	74.4	19.3	3.85:1
Fraction 1 and 2	607.4	192.6	3.15:1

Note:

The extracts of bacteroids were prepared as described in Figure 14 and in the text. Assays for acetylene reduction and nitrogen fixation are described under Materials and Methods. When fractions 1 and 2 were assayed together, 0.05 ml of each fraction (0.29 mg protein from fraction 1 and 0.20 mg from fraction 2) were utilized and the results expressed on the basis of the protein in fraction 2.

The nitrogenase system from nodule bacteroids not only reduces acetylene and nitrogen, but also catalyzes an ATP-dependent reduction of KCN and NaN_3, and in this respect behaves like the nitrogenase from Clostridium pasteurianum and Azotobacter vinelandii (Hardy and Knight, 1967a; Schöllhorn and Burris, 1967).

Detroy et al. (1968) have investigated the effect on nitrogenase activity of combining fractions from one organisms with fractions from another organism. Using the facultative anaerobic bacteria, Klebsiella pneumoniae and Bacillus polymyxa, the oligate aerobe, Azotobacter vinelandii, and the obligate anaerobe Clostridium pasteurianum, it was found that the facultative anaerobes exhibited a wide range of complementarity. Both the obligate aerobe and obligate anaerobe were less effective in their ability to cross with the facultative bacteria, and failed to show any complementarity to one another.

In view of these findings it was expected that the nitrogenase fractions from soybean nodule bacteroids and Azotobacter vinelandii would be complementary to one another. Experiments were conducted to separate the nitrogenase enzyme from Azotobacter vinelandii into two fractions (Kelley et al., 1967) and to combine these fractions with nitrogenase fractions previously obtained from bacteroids. In all cases the fractions from one organism demonstrated an effective cross with those of the other organism, although the combination between fraction 1 of the bacteroid nitrogenase and fraction 2 of Azotobacter vinelandii

exhibited the more effective combination. These results further demonstrate the similarities in the nitrogenase enzyme system, regardless of its source.

Large samples of bacteroid extract have been fractionated on DEAE-cellulose columns by use of Tricine buffer with increasing concentrations of NaCl to elute proteins. A brown fraction with an absorption peak at 418 ηm and containing about 0.07 μmoles of non-heme iron and 0.15 μmoles of labile sulfur per mg of protein has been isolated. Such preparations maintained under an atmosphere of argon lost 90 percent of their labile sulfur within five hours. This protein is of interest because the nitrogenase system from Clostridium pasteurianum (Mortenson, 1964, and 1966) and Azotobacter vinelandii (Bulen et al., 1966; Hinkson and Bulen, 1967; Yoch et al., 1969) have been shown to contain non-heme iron components. To date no definite role for this fraction in electron transport has been established. It would not function in typical assays for ferredoxin.

SUMMARY

The primary objective of this investigation was to obtain cell-free extracts from soybean nodules which were capable of fixing atmospheric nitrogen. Initial experiments with nodule brei indicated that the site of nitrogen fixation was located in the bacteroid. Subsequent experiments were conducted to determine the conditions necessary for obtaining cell-free extracts from nodule bacteroids capable of nitrogen fixation. Finally, some of the properties of the nitrogenase system from nodule bacteroids were examined followed by partial purification and further investigation of some of its physical properties. Throughout both the in vivo and in vitro investigation of the nitrogenase system from nodule bacteroids, acetylene reduction was used as an effective assay for nitrogen fixation. The results of these experiments are summarized as follows:

1. Intact soybean nodules were shown to reduce acetylene to ethylene at a linear rate for up to one hour. An optimum oxygen concentration of 0.2 atm and an optimum acetylene concentration of 0.1 atm were established for acetylene reduction by intact soybean nodules.
2. Soybean nodule breis and cell-free extracts capable of fixing nitrogen were prepared for the first time using strictly anaerobic conditions, a buffered ascorbate medium, and insoluble polyvinyl-polypyrrolidone to remove endogenous phenolics. The cell-free

extract contained the ability to fix nitrogen and to reduce acetylene, and required an ATP-generating system and $Na_2S_2O_4$ as a reductant for activity.

3. The optimum conditions for nitrogen fixation by cell-free extracts were examined. The optimum concentrations of $Na_2S_2O_4$ and creatine phosphate were 3.3 μmoles and 45 μmoles per ml of reaction mixture respectively. The rate of ammonia synthesis was found to be constant for a period of 60 minutes, and was directly proportional to enzyme concentrations between 0 and 21 mg of protein per reaction. In a series of experiments the mean K_m for nitrogen was found to be 0.056 ± 0.005 atm, with a V_{max} of 3.47 ηmoles of ammonia produced per mg of protein per minute.
4. An investigation of ATP-dependent hydrogen evolution during nitrogen fixation demonstrated the absence of a characteristic hydrogenase, and suggested that the nitrogenase system was responsible for hydrogen evolution. Additional experiments demonstrated that hydrogen functions as a competitive inhibitor of nitrogen fixation, with a K_i of 0.106 atm when the reactions contained 0.05 atm of hydrogen.
5. The nitrogenase system from bacteroid extracts was fractionated into two components by chromatography on a DEAE-cellulose column. Both fraction 1 and 2 were found to contain iron, with only fraction 1 containing molybdenum. Apparent S values for

the two components suggested a molecular weight between 100,000 and 150,000 for fraction 1 and 40,000 and 60,000 for fraction 2. Recombination of the two fractions in varying proportions was studied to obtain the maximum specific activity. Partial purification of the nitrogenase system has resulted in an increase in specific activity from 12 ηmoles of N_2 fixed per min per mg of protein in crude extract to 193 ηmoles of N_2 fixed per min per mg of protein in combined fractions, which indicates a 16-fold purification. Preliminary experiments suggest that the two components of the nitrogenase system from nodule bacteroids will effectively cross with the two nitrogenase fractions from Azotobacter vinelandii.

6. Reduction of nitrogen gas to ammonia by the nitrogenase system requires six electrons, while the reduction of acetylene to ethylene requires only two electrons. On the basis of this 3:1 ratio of electron requirements, the ratio between the rate of acetylene reduction and nitrogen fixation was examined at different stages of purification, and found to be about 3:1 as expected.

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