AN ABSTRACT OF THE THESIS OF

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Title: PURIFICATION AND SOME PROPERTIES OF NITROGENASE FROM SOYBEAN ROOT NODULES

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Abstract approved: Harold J. Evans

The Mo-Fe protein from bacteroids of soybean nodules has been purified to a high degree of homogeneity as judged by disc gel electrophoresis and ultracentrifugal analysis. The purification procedure included: polypropylene glycol fractionation, heat treatment and chromatography on DEAE-cellulose and Sephadex G-200. Specific activities of several preparations of the purified protein ranged between 850 and 1000 nmoles C_2H_2 reduced per minute per mg protein and 260 and 300 nmoles N_2 reduced per minute per mg protein. When the Mo-Fe protein was assayed with an optimum level of Fe protein, an apparent K_m for N_2 of 0.068 atm was obtained. The time for 50% inactivation of the protein under 0.2 atm of O_2 at 30°C is 4.5 minutes. A molecular weight of 197,600 for the protein was determined by low speed sedimentation equilibrium experiments and a molecular weight of 202,000 was determined by the high speed

sedimentation equilibrium method. Sedimentation equilibrium analysis of the protein in 6 M guanidine hydrochloride indicated one size of subunit with a molecular weight of about 50,000. Treatment of the protein with 2-mercaptoethanol and sodium dodecylsulfate followed by electrophoresis in gels containing sodium dodecylsulfate also produced one size of subunit. Ultracentrifugal analysis revealed a $s^{o}_{20,w}$ value of 9.99. A $\bar{v}$ of 0.732 ml per g was calculated from the amino acid composition. The ultraviolet spectrum of the protein exhibited an absorbance maximum of 279 nm and a molar extinction coefficient at this wavelength of 3.69×10^{5} cm^{-1} M^{-1}. A broad general absorbance was observed in the visible spectrum between 350 and 600 nm and no obvious shoulders or peaks were apparent. Analyses of samples of the protein revealed a mean of 1.3 Mo, 28.8 Fe, and 26.2 acid-labile sulfide atoms per molecule, based on a molecular weight of 200,000. All the common amino acids were present.

Purification and Some Properties of Nitrogenase from Soybean Root Nodules

by

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LIST OF ABBREVIATIONS

ATP	Adenosine-5'-triphosphate
ADP	Adenosine-5'-diphosphate
DEAE	Diethylaminoethyl
DNase	Deoxyribonuclease
K_i	Michaelis constant for inhibition
K_m	Michaelis constant
P_i	Inorganic phosphate
PVP	Polyvinylpyrrolidone
RNase	Ribonuclease
TES	N-tris (hydroxymethyl) methyl-2-amino-ethane sulfonic acid

PURIFICATION AND SOME PROPERTIES OF NITROGENASE FROM SOYBEAN ROOT NODULES

INTRODUCTION AND STATEMENT OF PROBLEM

Postgate (1968) has stated "Living things contain 10 to 15 percent of nitrogen--unless they are uncommonly fat or woody." Yet, all animals and most plants are unable to utilize directly the diatomic gas (N_2) which accounts for 78% by volume of the composition of dry air. The direct utilization of N_2 is restricted to a relatively small number of microorganisms which, individually or in symbiosis with certain higher plants, possess the ability to reduce N_2 to ammonia (NH_3). Most of the nitrogen found in living things is converted to a usable form by these N_2-fixing microorganisms.

Donald (1960) has estimated that 10^8 tons of N_2 are reduced to NH_3 on the Earth annually. A sizable portion of this fixation can be attributed to approximately 15,000 species of the Leguminosae which, in symbiosis with a number of species of the genus Rhizobium, reduce N_2 to NH_3. Burns and Hardy[1] have estimated that leguminous crops in the United States alone fix 7.6×10^6 metric tons of N_2 annually.

Concern over a world shortage of protein has stimulated basic and applied research on the process of biological N_2 fixation (Nutman, 1971). Microorganisms that fix N_2 are capable of converting inert

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[1]Personal Communication

nitrogen gas (N_2) to NH_3 under conditions that are conducive to life. In contrast, the major industrial process for producing NH_3, the Haber-Bosch process, requires a high temperature (500° C) and pressure (1000 atm). In a time when there is so much concern over the shortage of food protein and over the shortage of fossil fuels and the pollution caused by the use of these fuels, it seems of paramount importance to understand the mechanism by which N_2-fixing microorganisms accomplish the reduction of N_2.

An important event which contributed greatly to the understanding of this process was the preparation of extracts containing the N_2-fixing enzyme (nitrogenase) from Clostridium pasteurianum (Carnahan et al., 1960a, 1960b). The nitrogenase from Clostridium pasteurianum and Azotobacter vinelandii has subsequently been resolved into two components. Both of these components have been purified and their physical and chemical properties determined. Extracts containing nitrogenase have been prepared from legume bacteroids (Koch, Evans and Russell, 1967a, 1967b) and resolved into two components (Klucas et al., 1968 and Bergersen and Turner, 1970).

At the time this investigation was initiated, physical and chemical properties of highly purified components of nitrogenase from bacteroids of soybean nodules had not been determined. The primary purpose of this investigation, therefore, was to develop a procedure for the purification of the Mo-Fe protein component of nitrogenase

from bacteroids and to determine some of its important physical and chemical properties.

REVIEW OF LITERATURE

Nitrogenase from Free-Living Microorganisms

In 1960, the first cell-free extracts with reproducible, nitrogen-fixing activity were prepared from the anaerobic microogranism Clostridium pasteurianum by Carnahan et al. (1960a, 1960b). At about the same time, nitrogen fixation was claimed in a system using extracts of Azotobacter cells prepared in the medium in which they grew (Nicholas and Fisher, 1960). In these experiments, essential components including reductant and a source of ATP were not included. In 1964, a reproducible, cell-free system, in which nitrogenase activity of Azotobacter vinelandii was supported by hydrogenase and an ATP-generating system, was reported (Bulen, Burns and LeComte, 1964). Since the initial demonstration of N_2-fixing activity in cell-free extracts of Clostridium pasteurianum and Azotobacter vinelandii, nitrogenases from these organisms have been extensively studied.

Substrates of Nitrogenase

Early studies on whole cells of the N_2-fixing organisms, Clostridium pasteurianum and Azotobacter vinelandii, revealed that NH_3 is an intermediate in the fixation of N_2 (Zelitch et al., 1951 and Newton, Wilson and Burris, 1953). Ammonia also was shown to be the product of N_2 fixation by cell-free extracts of Clostridium

pasteurianum (Carnahan et al., 1960b). The reduction of N_2 in the presence of nitrogenase has been proposed to involve three 2-electron reduction steps. In these proposed steps, molecular nitrogen ($N \equiv N$) is reduced to the oxidation levels of diimide ($\overset{H}{\underset{|}{N}}=\overset{H}{\underset{|}{N}}$), then to hydrazine ($\overset{H}{\underset{|}{N}}-\overset{H}{\underset{|}{N}}$) and finally to $2NH_3$ (Hardy and Knight, 1967). No intermediates between N_2 and NH_3 have been isolated; and therefore the proposed diimide and hydrazine intermediates, if they exist, are enzyme-bound.

Lockshin and Burris (1965) observed that nitrous oxide (N_2O), carbon monoxide (CO), nitric oxide (NO) and hydrogen inhibit N_2 fixation by cell-free extracts of Clostridium pasteurianum. Azide (N_3^{1-}) and cyanide (CN^{1-}) were weak inhibitors of N_2 fixation in these extracts. Dilworth (1966) and Schöllhorn and Burris (1966) independently demonstrated acetylene (C_2H_2) inhibition of N_2 fixation in extracts of Clostridium pasteurianum.

Hardy and Knight (1966a) studied the substrate specificity of nitrogenase from Azotobacter vinelandii and Clostridium pasteurianum. They observed that nitrogenase catalyzes the reduction of N_2O to N_2 and H_2O, of HCN to CH_4 and NH_3 and of N_3^{1-} to NH_3 and N_2 as well as the reduction of N_2 to NH_3. Dilworth (1966) made the important observation that nitrogenase from Clostridium pasteurianum catalyzed the reduction of acetylene (C_2H_2) to ethylene (C_2H_4). Requirements for reduction of these inhibitors or alternate substrates were the same

as those for the reduction of N_2. It became apparent, therefore, that these inhibitors are actually nonphysiological substrates of nitrogenase and that their inhibition of N_2 fixation is due to competition for electrons.

Acetylene reduction coupled with gas chromatography was proposed as an effective assay for nitrogenase activity (Hardy and Knight, 1967). Since this initial proposal, the acetylene reduction assay has proven to be a convenient means of following nitrogenase activity during purification of the enzyme and determination of its catalytic properties.

Components of Nitrogenase

When it became possible to supply the reductant and ATP requirements for N_2 fixation in cell-free extracts by addition of exogenous electron donor systems and ATP generating systems, purification and the subsequent characterization of nitrogenase became feasible. Mortenson (1965) separated nitrogenase from Clostridium pasteurianum into two components by batchwise DEAE-cellulose treatment of a protamine sulfate supernatant fraction of a crude extract. Both components were required for nitrogenase activity. One component containing Mo and non-heme Fe, remained in the unadsorbed fraction while the second component, containing non-heme Fe, was adsorbed to the DEAE-cellulose. Bulen and LeComte (1966), using

DEAE-cellulose column chromatography, successfully separated nitrogenase from Azotobacter vinelandii into two components, one containing Mo and non-heme Fe and the second containing non-heme Fe. Since their initial separation, these two components have been given several different names. In this discussion, however, the component which contains Mo and non-heme Fe will be referred to as the Mo-Fe protein; and the component which contains non-heme Fe will be referred to as the Fe protein.

Purification - Nitrogenase from most sources is extremely labile to O_2, therefore all purification procedures are complicated by the necessity of manipulating the enzyme or its components under an atmosphere of argon (Ar), N_2 or H_2. Assays used to follow activity of nitrogenase or its components during purification include reduction of C_2H_2 to C_2H_4, N_2 to $2NH_3$ and $2H^+$ to H_2. The products of these reactions are readily measured by gas chromatography, Nesslers reagent and manometry, respectively. Catalysis of all three of these reactions is dependent upon reductant and ATP. Since each component is measured by recombining with an optimum of the complementary component, specific activities are expressed in terms of the limiting component.

Dalton et al. (1971) described a procedure for purification of large quantities of nitrogenase from Clostridium pasteurianum. Their procedure involved protamine sulfate fractionation of crude

extracts to separate the component proteins, two protamine sulfate precipitations and subsequent solubilizations of the Mo-Fe protein, and filtration of the Mo-Fe protein on 5.0 × 50 cm Sephadex G-100 columns. The Fe protein from the initial protamine sulfate fractionation was precipitated a second time with protamine sulfate, solubilized, chromatographed on Sephadex G-100 columns, precipitated with protamine sulfate a third time, and solubilized as described by Moustafa and Mortenson (1969). The Mo-Fe protein purified in this manner migrated as one band during electrophoresis in polyacrylamide gels and had a specific activity of 1200 nmoles C_2H_2 reduced per minute per mg protein. The Fe protein was estimated to be 90-95% pure by disc gel electrophoresis and ultracentrifugation and showed a specific activity of 2700 nmoles C_2H_2 reduced per minute per mg protein. The recovery of Mo-Fe protein and Fe protein activity by this method was 50% and 25% respectively. Tso, Ljones and Burris (1972) described a different procedure for the purification of nitrogenase proteins from Clostridium pasteurianum. Their method included two polyethylene glycol precipitations and chromatography on DEAE-cellulose, Sephadex G-100, and Sephadex G-200. Both proteins were purified 70-80 fold from the crude extract and each was essentially pure when tested by polyacrylamide disc gel electrophoresis under anaerobic conditions. Specific activities were 2500 and 3100 nmoles C_2H_2 reduced per minute per mg protein for the Mo-Fe protein and

Fe protein, respectively; and the overall yield of activity was 60% for each of the two proteins.

Nitrogenase from Azotobacter vinelandii has been purified (Burns, Holsten and Hardy, 1970). Crude extracts were treated with protamine sulfate to remove nucleic acids, heat treated at 60° C for 10 minutes to remove non-nitrogenase proteins, precipitated with protamine sulfate, solubilized, and then chromatographed on DEAE-cellulose to separate the component proteins. The Mo-Fe protein component was then crystallized from the DEAE-cellulose fraction by decreasing the ionic strength of the buffer. White, needle-shaped crystals were obtained which, upon solubilization, produced a brown solution with a specific activity of 1400 nmoles H_2 evolved per minute per mg protein. Recently, Shah, St. John and Brill (1973) modified the procedure of Burns et al. (1970) by heat treatment and subsequent chromatography of the Mo-Fe protein fraction on a second DEAE-cellulose column. Crystallization of the Mo-Fe protein purified in this manner produced brown, needle-shaped crystals which, upon solubilization, had a specific activity of 2500 nmoles C_2H_2 reduced per minute per mg protein. The protein was pure when tested by disc gel electrophoresis.

It has become necessary to consider the purity of nitrogenase components in terms of "active site purity" (Tso, Ljones, and Burris, 1972). Recently, Zumft et al. (1972) separated the Mo-Fe protein

from Clostridium pasteurianum, which appeared electrophoretically pure (Dalton et al., 1971) into two components by chromatography on long DEAE-cellulose columns. The component which was eluted from the column first was inactive in nitrogenase assay systems, but immunoprecipitin tests of the inactive protein were positive against Mo-Fe protein antiserum. The specific activity of the active component increased from 1200 to 2600 nmoles C_2H_2 reduced per minute per mg protein as a result of removing the inactive protein. These results indicate that the inactive component was derived from the Mo-Fe protein but, as a result of alteration, lacks the active site.

Properties of the Mo-Fe Protein. Nakos and Mortenson (1971a) reported that the Mo-Fe protein from Clostridium pasteurianum is composed of two sizes of subunits with molecular weights of 50, 000 and 60, 000. Huang, Zumft and Mortenson (1973) reported a native molecular weight of 220, 000 for the Mo-Fe protein from Clostridium and concluded that it is a tetrameric complex composed of two 50, 000 and two 60, 000 molecular weight subunits. The Mo-Fe protein from Azotobacter vinelandii has been shown to have a molecular weight in the range of 270, 000-300, 000 (Burns, Holsten and Hardy, 1970) and to consist of two sizes of subunits with molecular weights of about 40, 000 (Burns and Hardy, 1972).

The most highly purified Mo-Fe protein from Clostridium has been reported to contain 2 Mo, 22-24 Fe, and 22-24 acid labile sulfide

atoms per molecule based on a molecular weight of 220,000 (Zumft et al., 1972). The Mo-Fe protein from Azotobacter purified by the method of Burns and Hardy (1972) contained 2 Mo, 32-36 Fe, and 28 acid-labile sulfide atoms per molecule based on a molecular weight of 270,000. The Mo-Fe protein from both Clostridium and Azotobacter contains all the common amino acids (Burns and Hardy, 1972 and Mortenson, 1972).

The absorption spectrum of the Mo-Fe protein from Clostridium showed a typical absorption maximum at a wavelength of 280 nm and a broad general absorbance between 350 and 600 nm with no distinct peaks or shoulders (Dalton et al., 1971). The spectrum for the protein from Azotobacter also had a maximum absorbance at a wavelength of 280 nm and a distinct shoulder at 420 nm (Burns, Holsten and Hardy, 1970). Upon addition of $Na_2S_2O_4$, small peaks at 525 nm and 557 nm wavelengths were observed. The spectrum of more highly purified protein from Azotobacter lacks the shoulder and peaks in the visible region and is quite similar to that described for the protein from Clostridium (Shah, St. John and Brill, 1973). It now appears that the white crystals of the Mo-Fe protein from Azotobacter (Burns, Holsten and Hardy, 1970) were contaminated with cytochromes.

The EPR spectra of the Mo-Fe protein from Clostridium and Azotobacter have resonances at g-values of 2.01, 3.7, and 4.3

(Dalton et al., 1971 and Orme-Johnson et al., 1972). These resonances were observed at temperatures lower than 20°K. Purified Mo-Fe proteins from these organisms are labile to O_2 but indefinitely stable when stored at temperatures below -15° C under anaerobic conditions (Dalton et al., 1971 and Burns and Hardy, 1972).

Properties of the Fe Protein. The purified Fe protein from Clostridium has been found to be a dimer composed of two 27, 500 molecular weight subunits and to contain four Fe and four acid-labile sulfide atoms per dimer (Nakos and Mortenson, 1971b). The protein from Azotobacter is reported to have a molecular weight of about 40, 000 (Bulen and LeComte, 1966). The protein from both organisms is labile to O_2 and to cold (0° C) (Moustafa and Mortenson, 1969 and Burns and Hardy, 1972). At concentrations of 40 mg per ml, the protein can be stored for months in liquid N_2 with little loss in activity.

The purified Fe proteins from Clostridium and Azotobacter have rhombic EPR signals with g-values of 2.05, 1.94, and 1.89 in the reduced state and these signals are altered upon the binding of Mg-ATP (Orme-Johnson et al., 1972 and Zumft, Palmer and Mortenson, 1973). The Fe protein from these organisms has no definite EPR signal in the oxidized state (not O_2-denatured) (Orme-Johnson et al., 1972), and the protein from Clostridum binds two Mg-ATP molecules per dimer of 55, 000 (Zumft, Palmer and Mortenson, 1973).

Role of Reductant and ATP in Nitrogen Fixation

Demonstration of Requirements. Cell-free extracts prepared by Carnahan et al. (1960a, 1960b) from Clostridium pasteurianum required pyruvate. Study of the metabolic fate of pyruvate in these N_2-fixing systems resulted in elucidation of requirements for support of cell-free N_2-fixation.

An electron transfer protein, ferredoxin, was purified 60 fold from extracts of Clostridium pasteurianum and was shown to function in the degradation of pyruvate by the phosphoroclastic reaction (Mortenson, Valentine and Carnahan, 1962). Mortenson, Valentine and Carnahan (1963) demonstrated that ferredoxin functions in the transfer of electrons from pyruvate in the presence of pyruvic dehydrogenase and hydrogenase forming H_2 during the phosphoroclastic breakdown of pyruvate. Mortenson (1964a) demonstrated the absolute requirement of ferredoxin for support of N_2 fixation in extracts of Clostridium pasteurianum when pyruvate was used as electron donor. Removal of ferredoxin from crude extracts with DEAE-cellulose resulted in the loss of the capacity to degrade pyruvate and to fix N_2. Addition of purified ferredoxin to the ferredoxin-depleted extracts restored both capacities.

Hardy and D'Eustachio (1964) concluded that, in addition to a suitable electron donor, N_2 fixation in cell-free extracts of

Clostridium pasteurianum required high energy phosphate. Other investigators (Mortenson, 1964b and Bulen, Burns and LeComte, 1964) demonstrated that an electron donor system consisting of H_2, hydrogenase, and ferredoxin and an ATP-generating system consisting of Mg^{2+}, acetyl phosphate, acetate phosphotransferase or a second consisting of creatine phosphate, creatine phosphokinase, Mg^{2+} and a low level of ATP or ADP would support N_2 fixation catalyzed by extracts of Clostridium pasteurianum or Azotobacter vinelandii.

Bulen, Burns and LeComte (1965) discovered that sodium dithionite ($Na_2S_2O_4$) would support N_2 fixation in extracts of Azotobacter vinelandii in the absence of ferredoxin and methyl viologen when ATP was supplied by an ATP-generating system. Since this discovery, $Na_2S_2O_4$ has been employed extensively as a source of reductant in cell-free N_2 fixation.

Dilworth et al. (1965) concluded that Mg^{2+} is required for ATP utilization as well as for ATP generation. This conclusion was based on the observation that Mn^{2+} substituted very effectively for Mg^{2+} in the generation of ATP, but supported only 30% of the N_2-fixing activity observed in a reaction containing Mg^{2+}. Subsequent studies, which are discussed in another section, have provided more direct evidence for the involvement of Mg^{2+} in ATP utilization.

ATP-Dependent H_2 Evolution and Reductant-Dependent ATPase. Bulen, Burns and LeComte (1965) observed reductant ($Na_2S_2O_4$) and

ATP-dependent evolution of H_2, from reaction mixtures containing N_2-fixing extracts of Azotobacter vinelandii and incubated under N_2. This ATP-dependent H_2 evolution increased fourfold under an atmosphere of argon. Under N_2, fixation of N_2 occurred which consumed reductant and hence competed with H^+ for electrons. Hardy, Knight and D'Eustachio (1965) demonstrated ATP-dependent H_2 evolution from reaction mixtures containing N_2-fixing extracts of Clostridium pasteurianum when carbon monoxide (CO) was used to inhibit ATP-independent H_2 evolution from $Na_2S_2O_4$ via ferredoxin and hydrogenase.

Dilworth et al. (1965) observed a stimulation of acetyl phosphate consumption by reductant (H_2) in reactions catalyzed by N_2-fixing extracts of Clostridium pasteurianum and termed the phenomenon reductant-dependent elimination of P_i from ATP in N_2-fixing extracts of Azotobacter vinelandii. The presence of reductant-dependent ATPase and ATP-dependent H_2 evolution in N_2-grown but not NH_3-grown cells (Dilworth et al., 1965; and Hardy, Knight and D'Eustachio, 1965) indicated that these activities are a part of the N_2-fixing system.

Dilworth et al. (1965) demonstrated the insensitivity of reductant-dependent ATPase activity to levels of carbon monoxide (CO) that inhibited N_2 fixation and Hardy, Knight and D'Eustachio (1965) showed an insensitivity of ATP-dependent H_2 evolution to CO. From these results, these authors concluded that the site of N_2-chemisorption

upon nitrogenase is not involved in reductant-dependent ATPase or ATP-dependent H_2-evolving activity. The elimination of P_i from ATP was shown to occur during ATP-dependent H_2 evolution (Hardy and Knight, 1966b). Thus, ATP-dependent H_2 evolution and reductant-dependent ATPase represent two ways of measuring the same activity.

Bulen and LeComte (1966) found that both fractions of nitrogenase from Azotobacter vinelandii separated on DEAE-cellulose are required for N_2 fixation, ATP-dependent H_2 evolution and reductant-dependent ATPase activity. Similar results were obtained when partially purified nitrogenase components of Clostridium pasteurianum were combined and assayed (Kennedy, Morris, and Mortenson, 1968).

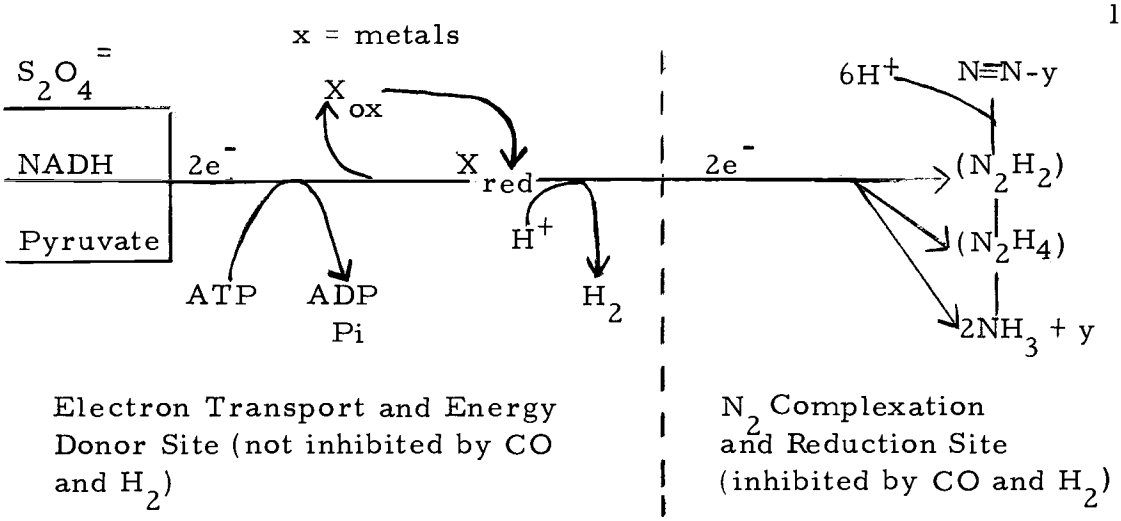
Stoichiometry of ATP Utilization Versus Electron Transfer. Hardy and Knight (1966b) reported the hydrolysis of two ATP molecules for each H_2 evolved in reactions containing N_2-fixing extracts of Azotobacter vinelandii. A minimum value of three was obtained for the ratio of ATP hydrolyzed per two electrons transferred for the N_2-fixing reaction catalyzed by extracts from Clostridium pasteurianum at pH 6.6 (Jeng, Morris and Mortenson, 1970). The ratio, however, varied between three and eight, depending on the pH of the assay system. Hadfield and Bulen (1969) obtained a ratio of five ATP molecules hydrolyzed per two electrons transferred in reduction catalyzed by the nitrogenase complex of Azotobacter vinelandii. Ljones and Burris (1972) studied the stoichiometry of

ATP utilization per two electrons transferred using spectrophotometric determination of $Na_2S_2O_4$ oxidation as a measure of total electron transfer. They observed ratios which ranged from four with an excess of Fe protein to 20 with an excess of Mo-Fe protein. Four to five ATP's consumed per electron pair transferred under optimal conditions seems to be the most reproducible stoichiometry catalyzed by cell-free extracts containing nitrogenase.

Hadfield and Bulen (1969) concluded that ATP is hydrolyzed in two ways, only one of which leads to electron transfer, and that the concept of single-reaction stoichiometry is not applicable to this system. ATP has been proposed to bring about a conformational change in the nitrogenase molecule which is necessary for binding and subsequent reduction of substrates (Bulen et al., 1965). This process may not be directly coupled to electron transfer in the nitrogenase complex and may account for the high stoichiometry of ATP consumed per electron pair transferred.

Mechanism of N_2 Fixation

Based on the observation that CO inhibits the reduction of N_2 but not reductant-dependent ATP hydrolysis or ATP-dependent H_2 evolution, Hardy and Knight (1966b) proposed the following mechanism for the nitrogenase reaction:



The site of N_2 complexation and reduction is distinctly separated from the sites of ATP hydrolysis and H^+ reduction since CO inhibits NH_3 formation but not ATP hydrolysis or H_2 evolution. Recent inhibitor studies (Hwang, Chen, and Burris, 1973) have indicated that the nitrogenase complex may have as many as five modified sites for the binding of electron acceptors or inhibitors.

Bui and Mortenson (1968), by use of gel equilibration techniques, have shown that the Fe protein of nitrogenase from Clostridium pasteurianum binds ATP and ADP and that the binding of ATP requires Mg^{2+}. These investigators also obtained evidence that indicated preferential binding of the substrate (CN^{1-}) to the Mo-Fe protein. Reductant was not required for the binding of Mg-ATP to the Fe protein or for the binding of CN^{1-} to the Mo-Fe protein. Consequently, Bui and Mortenson (1968) proposed that the point of entry of electrons into the nitrogenase complex may be at the site of a Mg-ATP· Fe protein complex or a Mo-Fe protein· N_2 complex or a

combination of these two complexes.

Recent EPR studies on both components of the nitrogenase complex have supported the report of Bui and Mortenson (1968). Mg-ATP has been shown to convert the rhombic EPR signal of reduced Fe protein with g-values of 2.05, 1.94, and 1.89 to an axial signal with g-values near 2.05 and 1.94 (Orme-Johnson et al., 1972 and Zumft, Palmer and Mortenson, 1973). This alteration of the EPR signal is good evidence for the binding of Mg-ATP to the Fe protein.

The oxidized form of the Mo-Fe protein (not O_2-denatured) has an EPR signal with g-values at 4.3, 3.7, and 2.0 (Orme-Johnson et al., 1972). These researchers have observed that the signals of the Mo-Fe protein and Fe protein are additive when they are mixed in the presence of $Na_2S_2O_4$. When Mg-ATP is added to these mixtures, the EPR signal of the Mo-Fe protein is diminished by about 92%. This is interpreted to mean that electron transfer from the Fe protein to Mo-Fe protein commences upon the binding and subsequent hydrolysis of Mg-ATP. When reductant is depleted, the Fe protein loses its EPR signal; and the Mo-Fe protein regains its signal. Both events are attributed to oxidation of the system (Orme-Johnson et al., 1972.

The results of Bui and Mortenson (1968) and the EPR data (Orme-Johnson et al., 1972 and Zumft, Palmer and Mortenson, 1973)

are consistent with the proposal that only the Mg-ATP· Fe protein complex is capable of transferring electrons to the Mo-Fe protein which reduces bound substrates. More direct evidence for the binding of substrate to the Mo-Fe protein is needed. It has not been established whether hydrolysis of Mg-ATP occurs during electron transfer from the Fe protein to the Mo-Fe protein or whether it occurs during the reduction of substrate. It is possible that the hydrolysis of Mg-ATP occurs in both steps.

Symbiotic Nitrogen Fixation

Site of N_2 Fixation

Aprison and Burris (1952), using the ^{15}N technique, established that excised soybean nodules fix N_2 for a period of about two hours after excision. The rate of N_2 fixation was greatly reduced in sliced nodules, and fixation was virtually lost when the nodules were crushed (Aprison, Magee, and Burris, 1954). Bergersen (1966a, 1966b) reported the preparation of N_2-fixing and H_2-evolving breis of soybean nodules. The N_2-fixing system of the breis required O_2 in order to function, but during the preparation of the breis it was necessary to maintain anaerobic conditions. Subsequently, the nodule breis were separated into bacteroid, soluble and membrane fractions; and it was shown that N_2-fixation and H_2 evolution are associated with

the bacteroid fraction (Bergersen and Turner, 1967). Addition of soluble and membrane fractions to the bacteroids failed to stimulate either activity. Koch, Evans and Russell (1967a) independently demonstrated that the N_2-fixing enzyme system is in the bacteroids. These investigators used anaerobic conditions for preparation of nodule breis and bacteroids and included PVP in the buffer to remove phenolic compounds released from the nodules during maceration. This greatly enhanced the C_2H_2-reducing capacity of nodule breis and bacteroid fractions and proved to be a more reproducible system than the system used by Bergersen and Turner (1967).

Cell-Free Extracts

Preparation of crude extracts of bacteroids from legume nodules lagged behind that of free-living microbes. Koch, Evans and Russell (1967a) prepared cell-free extracts of soybean nodule bacteroids which consistently catalyzed the reduction of C_2H_2 and N_2. This activity was dependent upon an ATP-generating system and reductant. The use of PVP to remove phenolics during maceration of nodules proved to be a crucial part of the preparation procedure. Little or no activity was measured when PVP was omitted. The strict exclusion of O_2 also was necessary for preparation of active extracts.

The optimum conditions for catalysis of N_2 reduction by the partially purified nitrogenase system of bacteroids were determined,

and other properties of the system studied (Koch, Evans and Russell, 1967b). The bacteroid nitrogenase system was reported to be saturated at a pN_2 of 0.25 atm, at a $Na_2S_2O_4$ concentration of 3.3 μmoles per ml and at a creatine phosphate concentration of 45 μmoles per ml. The bacteroid nitrogenase preparation also was shown to catalyze reductant and ATP-dependent H_2 evolution, and H_2 competitively inhibited the rate of N_2 fixation in this system. The K_i for H_2 was 0.016 atm.

Purification and Properties of Nitrogenase Components

Klucas et al. (1968) separated the partially purified nitrogenase system of bacteroids from soybean nodules into a Mo-Fe protein fraction and an Fe protein fraction using DEAE-cellulose. The Mo-Fe protein fraction had no catalytic activity alone, while the Fe protein exhibited low activity which was attributed to residual Mo-Fe protein. When the Mo-Fe protein and Fe protein fractions were combined and assayed, a specific activity for N_2 of 192 nmoles reduced per minute per mg of added Fe protein was obtained. Both components were stable when stored in liquid N_2, and both components were inactivated by exposure to O_2.

Bergersen and Turner (1970) also separated the nitrogenase system of bacteroids into two fractions, using filtration on Sephadex G-200 columns. The components prepared in this manner had

variable specific activities. Using the partially purified material, these investigators obtained a molecular weight for the Mo-Fe protein of 182, 000 and a molecular weight of 51, 000 for the Fe protein by gel filtration techniques. They also reported that the Mo-Fe protein contained 0.9 g-atoms of Mo and 8.7 g-atoms of Fe per mole (182, 000 mol. wt.) and that the Fe protein contained 0.9 g-atoms of Fe per mole (51, 000 mol. wt.).

Role of Leghemoglobin in Symbiotic N_2 Fixation

Many investigators have noted a positive correlation between the capacity of legume nodules to fix N_2 and the leghemoglobin content of the nodules (Virtanen, Erkama, and Linkola, 1947 and Bergersen, 1961). For some time, leghemoglobin was thought to participate directly in the N_2-fixing mechanism of legume nodules. This idea was proven incorrect when it was shown that bacteroids washed essentially free of leghemoglobin retained a capacity to fix N_2 or reduce C_2H_2 (Bergersen and Turner, 1967 and Koch, Evans and Russell, 1967a).

Imamura, Riggs and Gibson (1972) have demonstrated that leghemoglobin is one-half saturated with O_2 at a partial pressure of 0.047 mm Hg. The kinetic dissociation constant was shown to be 11 s^{-1} at 25°, a value which is quite similar to that of 10 s^{-1} for mammalian myoglobins. This close similarity caused these authors to suggest

that the primary role of leghemoglobin may be to facilitate O_2 diffusion within the legume nodule. Similar results were obtained by Wittenberg, Appleby and Wittenberg (1972); and these authors suggested that leghemoglobin may facilitate movement of O_2 and may serve to flatten the O_2 gradient from the periphery to the center of the nodule system. Bergersen and Turner (1968) have shown that moderate partial pressures of O_2 abolish N_2 fixation by isolated bacteroids. The results of physical studies indicate that leghemoglobin is capable of providing O_2 at the respiratory sites on the membranes of the bacteroids at partial pressures of O_2 that are not detrimental to the N_2-fixing system.

Recently, Bergersen, Turner and Appleby (1973) have designed experiments to test the suggested physiological role of leghemoglobin in soybean nodules. The evolution of H_2 by the intact nodules was inhibited when the nodules were pre-equilibrated with mixtures of argon and CO. Evolution of H_2 by the nitrogenase system in a bacteroid suspension was not inhibited by CO. From this it is suggested that the binding of CO with leghemoglobin of the intact nodule prevents O_2 binding and subsequent diffusion of O_2 to the respiratory sites that presumably are located on the membranes of the bacteroids. In other experiments, addition of purified leghemoglobin to dense suspensions of bacteroids (15-20% v/v) greatly enhanced H_2 evolution and C_2H_2 reduction by the nitrogenase system. These experiments convincingly

support facilitated O_2 diffusion as the primary role of leghemoglobin in symbiotic N_2 fixation.

EXPERIMENTAL PROCEDURE

Materials

TES (N-tris[hydroxymethyl]methyl 2-aminoethane sulfonic acid), dithiothreitol, 2-mercaptoethanol, creatine phosphokinase, DL-asparagine, and Na_2ATP were purchased from Sigma Chemical Co., (St. Louis, Mo.). Creatine phosphate was purchased from Pierce Chemical Co., (Rockford, Ill.); Napthol Blue Black from Allied Chemical Corp., (New York, N. Y.); polypropylene glycol (P-400) from Matheson Coleman and Bell, (Cincinnati, Ohio); and sodium dodecylsulfate from Eastman Kodak Company (Rochester, N. Y.). Sephadex G-100 and G-200 was purchased from Pharmacia Fine Chemicals (Uppsala, Sweden); Biogel (A-0. 5m) and Biogel (P-30) from Bio-Rad Laboratories (Richmond, Calif.); and DEAE-cellulose (Whatman DE-52) from H. Reeve Angel Inc. (Clifton, N. J). Sodium dithionite (98. 7%) was kindly supplied by Professor Joseph Chatt, ARC Unit of Nitrogen Fixation, University of Sussex, Brighton, England. High purity N_2 and Ar (8 ppm O_2 or less) were purchased from National Cylinder Gas Company (Portland, Ore.) and were further purified to remove traces of O_2 by the method of Lux (1959). C_2H_2 was generated by hydrolysis of technical grade calcium carbide. Other chemicals utilized were of the highest purity available.

Stock solutions of creatine phosphate, creatine phosphokinase, and Na_2ATP were prepared in 0. 025 M TES buffer, pH 7. 5. A stock solution of 0. 2 M $Na_2S_2O_4$ in deoxygenated 0.05 M TES buffer, pH 8.0,

was prepared just before use.

Analytical Methods

Nitrogenase Assays

Reaction mixtures used in acetylene reduction experiments contained 50 μmoles of creatine phosphate, 7.5 μmoles of Na_2ATP, 0.2 mg creatine phosphokinase, 20 μmoles of $Na_2S_2O_4$, 10 μmoles of $MgCl_2$ and 37.5 μmoles of TES buffer, pH 7.5, in a total volume of 1.5 ml. All components of the reaction mixture except $Na_2S_2O_4$ and nitrogenase or its components were placed in 21 ml serum bottles fitted with rubber serum stoppers The bottles were then evacuated and flushed eight times with Ar. Following the final evacuation, 0.9 atm of Ar was added and nitrogenase or its components and $Na_2S_2O_4$ were then transferred to the serum bottles by use of hypodermic syringes that previously had been flushed with N_2. Reactions were initiated by addition of 0.1 atm of C_2H_2, and the reaction mixtures were incubated in a 30° C water bath for 20 minutes on reciprocating shaker and were terminated by the addition of 0.5 ml of 50% (w/v) trichloroacetic acid. The C_2H_4 produced was measured by the gas chromatographic procedure described by Burris (1972).

The reaction components and assay conditions for measurement of N_2 reduction were the same as those used for acetylene reduction

with the exception that the Ar-C_2H_2 mixture was replaced by N_2 in the gas phase and reactions were initiated by addition of nitrogenase or its components. After incubating for 20 minutes, the serum stoppers were removed, saturated K_2CO_3 (2.0 ml) was added and ammonia (NH_3) determined (Burris, 1972).

One unit of N_2-reducing activity (N_2) is defined as the amount of enzyme required to reduce one nmole of N_2 to $2NH_3$ per minute and one unit of C_2H_2-reducing activity (C_2H_2) as the amount required to catalyze the formation of one nmole of C_2H_4 from C_2H_2 per minute. Activity of the separated protein components was determined in the presence of an optimum amount of the complementary component and specific activity was based on the concentration of the limiting component. Due to extreme lability, the Fe protein was limiting in crude extracts, and in solubilized and heat-treated polypropylene glycol precipitates. When such extracts were assayed, an optimum amount of Fe protein from DEAE columns was added in order to determine the true Mo-Fe protein activity. The protein content of the added Fe protein, however, was not included in specific activity calculations.

Electrophoresis

Analytical disc gel electrophoresis was performed in 6% polyacrylamide at pH 7.9 as described by Hedrick and Smith (1968). Twenty to 40 μg of purified Mo-Fe protein was electrophoresed at

5-7° C at a current density of 3 to 4 ma per tube. Samples of protein used in these experiments were placed in 2 mM mersalyl in order to minimize O_2 damage during electrophoresis. The subunit molecular weight of the purified Mo-Fe protein was determined by treatment with 1% sodium dodecylsulfate and 1% 2-mercaptoethanol and subsequent electrophoresis in polyacrylamide gels containing 0.1% sodium dodecylsulfate as described by Weber and Osborn (1969). The electrophoresis apparatus was purchased from the Canalco Co. (Bethesda, Md.) and the constant voltage power supply from Buhler Instruments, Inc. (Fort Lee, N. J.). Gels were polymerized in 0.5 × 7.0 cm glass tubes, stained with 0.17% Napthol Blue Black in 7.5% (v/v) acetic acid and 5% (v/v) methanol for one hour and destained overnight by diffusion in 7.5% (v/v) acetic acid and 5% (v/v) methanol.

Protein Determination

The protein content of samples containing more than 10 mg per ml was estimated by the biuret method of Gornall, Bardawill and David (1949). The protein content of samples containing less than 10 mg per ml were determined by the method of Lowry et al. (1951). Bovine serum albumin was used as a standard. The mean protein content of three samples of the most purified Mo-Fe protein fraction determined by dry weight measurements and by amino acid analysis was 75% of the value determined by the Lowry method (1951). In all

experiments in which the purified Mo-Fe protein (Table I) was used, the protein content was corrected to a dry weight basis.

Molybdenum, Iron, Acid-Labile Sulfide and Amino Acid Analyses

Samples of extracts were digested by the procedure of Bulen and LeComte (1966) and iron content of each sample was determined by the o-phenanthroline procedure of Ballentine and Buford (1957) and molybdenum content by the dithiol procedure of Clark and Axley (1955). The acid-labile sulfide content of undigested protein samples was assayed by the procedure of Fogo and Popowsky (1949).

The amino acid analyses of acid hydrolysates were performed according to the procedures of Spackman, Stein and Moore (1958) with a Spinco model 120B amino acid analyzer. Tryptophan was determined by the spectrophotometric method of Edelhoch (1967), and cysteine and cystine were determined as cysteic acid by the method of Moore (1963).

Spectrophotometry

A Cary model 11 recording spectrophotometer was used to record the ultraviolet and visible absorption spectra of the purified Mo-Fe protein. Samples suitable for determination of the ultraviolet spectrum were prepared by passing a solution containing 2 to 4 mg of

purified protein over a 1.5 × 25 cm column of Biogel P-30 equilibrated with an anaerobic solution containing 0.25 M NaCl in 0.02 M TES buffer, pH 7.5, 1 mM dithiothreitol and 0.06 mM $Na_2S_2O_4$. Buffer for use as a reference in the spectrophotometer was collected anaerobically just prior to the elution of the protein. The protein was collected anaerobically, and both reference buffer and protein sample were transferred to a glove box filled with N_2 and placed into 3 ml cylindrical quartz cuvettes with a 1 cm light path. The cuvettes were capped in the glove box and then removed, and the ultraviolet spectra were recorded.

Visible spectra were recorded on samples obtained directly from gel filtration columns after appropriate dilution with anaerobic 0.25 M NaCl in 0.02 M TES, pH 7.5, containing 1 mM dithiothreitol. Glass cuvettes (3 ml, 1 cm light path) modified so that the tops could be fitted with rubber serum stoppers were flushed 15 minutes with purified Ar or N_2. Solutions of the purified Mo-Fe protein were then transferred with hypodermic syringes to the anaerobic cuvettes. The absorbance of experimental samples was determined by use of a reference cuvette containing a solution of 0.02 M TES buffer, pH 7.5, 0.25 M NaCl, 1 mM dithiothreitol. After recording the spectra, aliquots of the samples were removed and protein contents were determined.

Sedimentation Experiments

A Spinco model E ultracentrifuge, equipped with the RTIC temperature control unit, electronic speed control, photoelectric scanner and monochromator was used for sedimentation studies. Sedimentation equilibrium experiments were conducted in 12 mm cells equipped with sapphire windows and either a six-channel or double sector centerpiece. For speeds below 15,000 rpm an An-J rotor was used. Above this speed, the An-D rotor was employed. Rayleigh interference patterns were recorded on Kodak type II G spectroscopic plates and absorbance at 450 nm (A_{450}) was recorded with the photoelectric scanner attachment. Fringe patterns were measured with a Nikon model 6 C microcomparator equipped with a digitizer (L. and W. Electronics, Dallas, Texas), which recorded the data on punched tape. The data were analyzed with a computer and a program written by Dr. R. Dyson.[2] Low speed equilibrium experiments were conducted as described by LaBar (1965). The time to attain equilibrium was shortened by using the techniques described by Richards, Teller and Schachman (1968). After reaching equilibrium at low speed, the rotor was accelerated and allowed to reach equilibrium at a speed sufficient to satisfy the high speed conditions of Yphantis (1964). This procedure permits determining the absolute fringe position necessary for

[2]Personal Communication

analyzing the results of the low speed run and simultaneously obtaining the data for a high-speed sedimentation equilibrium experiment. Sedimentation velocity experiments were conducted in 12 mm cells with double sector centerpieces. Concentration distribution (A_{280}) was recorded with the photoelectric scanner attachment.

The centrifuge cells were assembled, cooled to 0 to 2° C, flushed with prepurified N_2 for 15 minutes, then placed in a glove box under N_2, and filled with the appropriate solutions and sealed. These precautions were necessary to avoid artifacts caused by low levels of O_2 (see Discussion).

Purification Procedure

Anaerobic Techniques

All steps in the preparation of bacteroids and the subsequent purification of nitrogenase were carried out under conditions that minimize contact with O_2. Some operations involved in the preparation of nitrogenase were performed in a glove box filled with N_2 and containing an exposed solution (about 200 cm^2 open surface) of alkaline pyrogallol. Extracts containing nitrogenase were handled outside the glove box in sealed centrifuge tubes. Any transfers were made by use of hypodermic syringes which were purged with N_2 just prior to use. All buffers used in this procedure were sparged with

N_2 for 45 minutes or longer and 1.2 mM $Na_2S_2O_4$, unless otherwise stated, was added just prior to use.

Polypropylene glycol was heated to about 100° C and then sparged with N_2 for three to four hours as it cooled. The treatment of crude nitrogenase preparations with polypropylene glycol was carried out in 250 ml filter flasks which were evacuated and flushed with purified N_2 (Lux, 1959). The evacuation and flushing was repeated three times and then the flasks were flushed continuously with the purified N_2 (Lux, 1959) during the transfer and treatment of the preparations. The anaerobic techniques used in the column chromatography were essentially those described by Munson, Dilworth and Burris (1965).

Source and Preparation of Bacteroids

Soybean plants (Glycine max Merr. var. Chippewa) were inoculated, cultured, and nodules harvested and stored as described by Evans, Koch and Klucas (1972). Bacteroids were prepared essentially as described previously (Evans, Koch and Klucas, 1972) with the exception that the operation was scaled-up sufficiently to accommodate 450 g of nodules in each preparation.

Crude Extracts

Bacteroid suspensions (equal weights of bacteroids and 0.1 M TES pH 8.5) were ruptured in a precooled Aminco French press at

a pressure of 16,000 lb/in^2. The ruptured cells were collected under a stream of N_2 in polycarbonate tubes each of which contained 0.05 mg RNase and 0.005 mg DNase. After collecting the ruptured cells, the tubes were capped under a stream of N_2, and then centrifuged for one hour at 0 to 2° C at 48,000 × g. The supernatant fractions (50-60 ml) containing the crude nitrogenase (40-45 mg of protein per ml) were stored in liquid N_2 until further treatment.

Polypropylene Glycol and Heat Treatment

Cold polypropylene glycol was added dropwise to the vigorously stirred extract in a filter flask to obtain a concentration of 21% by volume. After addition of the polypropylene glycol, the solution was stirred an additional five minutes, transferred to polycarbonate tubes, and centrifuged for 15 minutes at 48,000 × g at 0 to 2° C. The pink pellet was discarded and the supernatant fraction containing the nitrogenase was transferred to another filter flask. The polypropylene glycol concentration was increased to 41% based on the original volume of extract and after five minutes the preparation was centrifuged at 30,000 x g for 15 minutes at 0 to 2° C. The supernatant fraction was discarded and the pellet containing both components of nitrogenase was dissolved in 18-20 ml of 0.1 M TES, pH 7.9. Aliquots (10 ml) of the solution were placed in stainless steel centrifuge tubes at room temperature and treated at 55° C for 2.5 minutes. The tubes were cooled

rapidly by plunging in an ice bath and then were centrifuged at 48,000 × g for 20 minutes at 0 to 2° C. The pellets were discarded and the supernatant fraction containing both nitrogenase components was stored in liquid N_2 until further purification. This fraction is referred to as preparation II (Table I).

DEAE-cellulose Chromatography

The DEAE-cellulose (DE-52) column (2.5 × 13.0 cm) was equilibrated with 800 ml of 0.025 M TES buffer, pH 7.9 which contained 0.6 mM $Na_2S_2O_4$ and 1 mM dithiothreitol. Three elution buffers were used in this chromatography procedure. These solutions contained 0.05 M NaCl, 0.15 M NaCl and 0.05 M $MgCl_2$ respectively. Each was prepared in sparged 0.1 M TES buffer, pH 7.9, and to each was added 0.6 mM $Na_2S_2O_4$ and 1 mM dithiothreitol just prior to use. The temperature of the column was maintained at 9° C by use of an external water jacket connected to a temperature-controlled water bath. The column was developed at a flow rate of about 60 ml per hour.

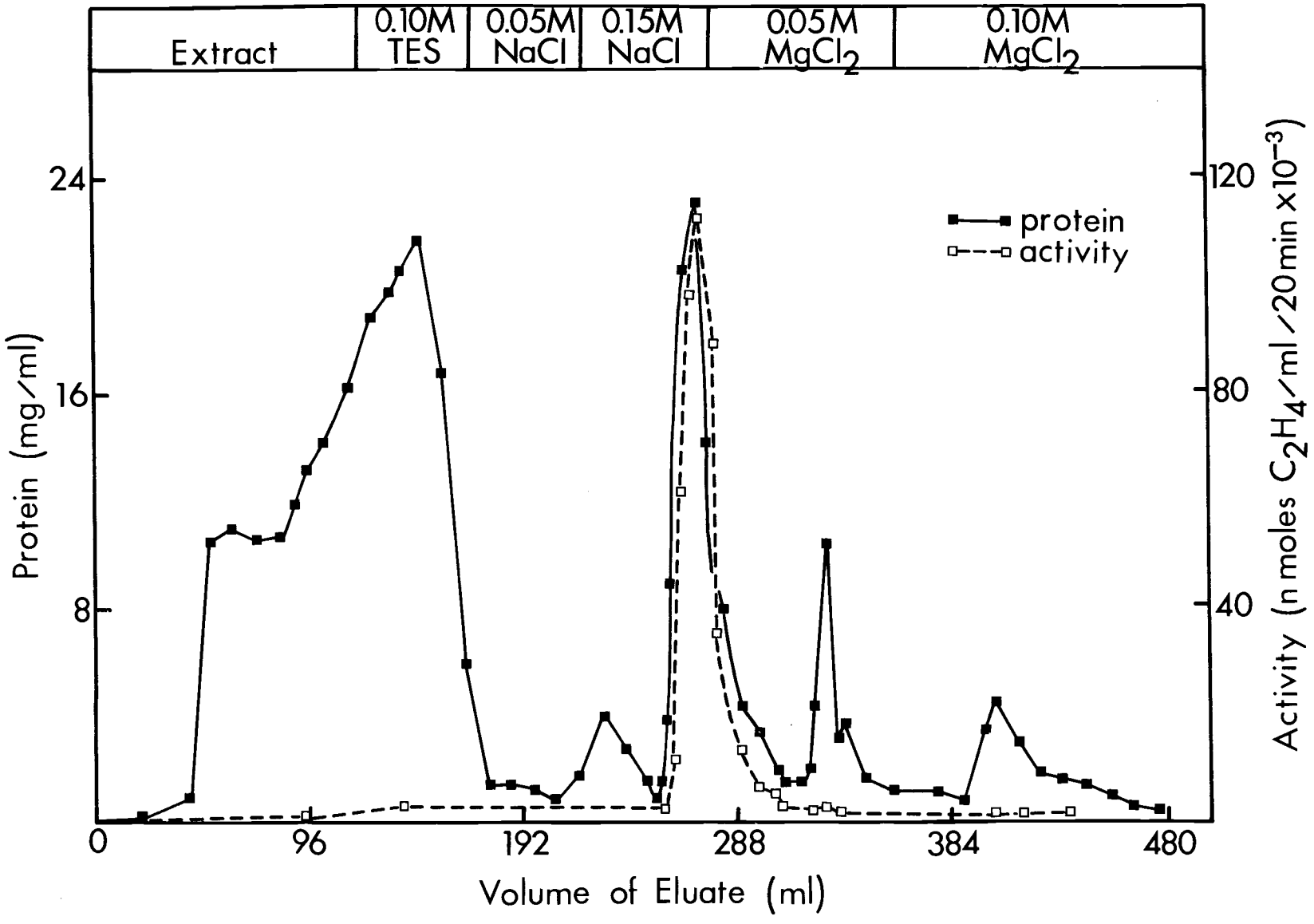
In a typical experiment, 32 ml of preparation II (Table I) (26 mg protein per ml) were placed on the column. The addition to the column of 80 ml of 0.05 M NaCl elution buffer removed considerable amounts of contaminating proteins. The Mo-Fe protein with a specific activity (C_2H_2) of 448 (preparation III, Table I) was eluted with 0.15 M NaCl

elution buffer and collected in the 161 to 169.5 ml fraction (tabulation of elution volume was initiated when enzyme was applied to the column). After elution of the Mo-Fe protein, the column was washed with an additional 75 ml of the 0.15 M NaCl elution buffer. The Fe protein with a specific activity (C_2H_2) of 500 was eluted with the 0.05 M $MgCl_2$ elution buffer and collected in the 292-298 ml fraction. Since both protein components are brown in appearance, they were followed visually on the column. Each nitrogenase component was assayed for C_2H_2-reducing activity after recombining with an optimum of the complementary component and for protein by the Lowry method (1951). Each component was stored in liquid N_2 until used.

In some experiments, crude extracts were heat-treated at 55°C for three minutes; and the supernatant fraction was used directly for DEAE-cellulose chromatography. In these experiments, the polypropylene glycol treatment was omitted. After further purification by chromatography on Sephadex G-200 or Biogel A-0.5m, the specific activity of the Mo-Fe protein was about the same as that of those preparations in which the polypropylene glycol treatment was included.

The elution profile illustrated in Figure 1 was obtained when an extract (heated at 55°C for three minutes) was chromatographed on a column of DEAE-cellulose. Mo-Fe protein with a specific activity (C_2H_2) of 250 was eluted from the column with the 0.15 M NaCl elution buffer. The protein was collected in the column effluent

Figure 1. Chromatography of a heat-treated nitrogenase preparation on DEAE-cellulose. A crude extract which had been heat-treated at 55° C for three minutes (120 ml, 28 mg protein per ml) was applied to a 2.5 × 13 cm DEAE-cellulose column and eluted at a flow rate of 60 ml per hour. Samples were assayed for C_2H_2-reducing activity as described in the Experimental Procedure section and for protein by the biuret method (Gornall, Bardawill and David, 1949). The activity plotted in the figure represents Mo-Fe protein activity determined in the presence of an optimum amount of Fe protein. The Fe protein used in the assays was collected in the effluent fraction between 320 and 333 ml.


Extract
0.10M TES
0.05M NaCl
0.15M NaCl
0.05M $MgCl_2$
0.10M $MgCl_2$
protein
activity
Protein (mg/ml)
Activity (n moles C_2H_4/ml/20min x10^{-3})
24
16
8
120
80
40
0
96
192
288
384
480
Volume of Eluate (ml)

between 260 and 280 ml. By use of the 0.05 M $MgCl_2$ elution buffer, the Fe protein was removed from the column in the effluent fraction collected between 320 and 333 ml. The non-heme iron protein that previously had been shown to function in the transfer of electrons from photosystem I of spinach chloroplast fragments to nitrogenase (Koch et al., 1970), was removed from the column in the 400 to 420 ml effluent by use of the 0.1 M $MgCl_2$ elution buffer. This procedure has the advantage that the two nitrogenase components and the non-heme iron protein, all of which are involved in the N_2-fixing process of bacteroids, are separated in one chromatographic procedure.

Sephadex G-200 Chromatography of the Mo-Fe Protein

The fraction which contained Mo-Fe protein from the DEAE-cellulose chromatography of the polypropylene glycol and heat-treated extract was concentrated to about 45 mg of protein per ml under N_2 by use of an Amicon ultrafiltration cell, equipped with an XM-100A membrane (Amicon Corp., Lexington, Mass.). The concentrated protein (2 ml) was applied to a 1.5 × 83 cm column of Sephadex G-200 that previously had been equilibrated with 500 ml of 0.25 M NaCl in 0.02 M TES, pH 7.5, containing 1 mM dithiothreitol and maintained at 5-7° C in a cold room. The protein was eluted at 5-7° C with the equilibration buffer using a flow rate of 10 ml per hour. Aliquots (3 ml) were collected and assayed for C_2H_2-reducing activity in the presence

of an optimum level of added Fe protein from DEAE-cellulose and for protein by the Lowry method (1951). The fraction of the Mo-Fe protein with a specific activity (C_2H_2) of 933 (Table I) was collected between 57.0 and 69.5 ml (Figure 2). This fraction was stored in liquid N_2 until used.

Sephadex G-100 Chromatography of the Fe Protein

The fraction of Fe protein from the DEAE-cellulose chromatography of the polypropylene glycol and heat-treated extract was further purified on a 2.5 × 43 cm column of Sephadex G-100. Before chromatography, the fraction was concentrated under N_2 to 20 mg of protein per ml by use of an Amicon ultrafiltration cell equipped with an XM-50 membrane. The column was equilibrated and developed with 0.05 M TES buffer, pH 8.0, containing 3 mM dithiothreitol, 3 mM $MgCl_2$ and 3 mM $Na_2S_2O_4$. The temperature was maintained at 15° C and the column was developed at a flow rate of 30 ml per hour. Aliquots were collected and assayed for C_2H_2-reducing activity in the presence of an optimum amount of Mo-Fe protein and for protein by the Lowry method (1951). Fe protein with a specific activity (C_2H_2) of about 600 was collected in the fraction between 93 and 103 ml. This fraction was stored in liquid N_2 until used. Although this procedure removed residual Mo-Fe protein activity

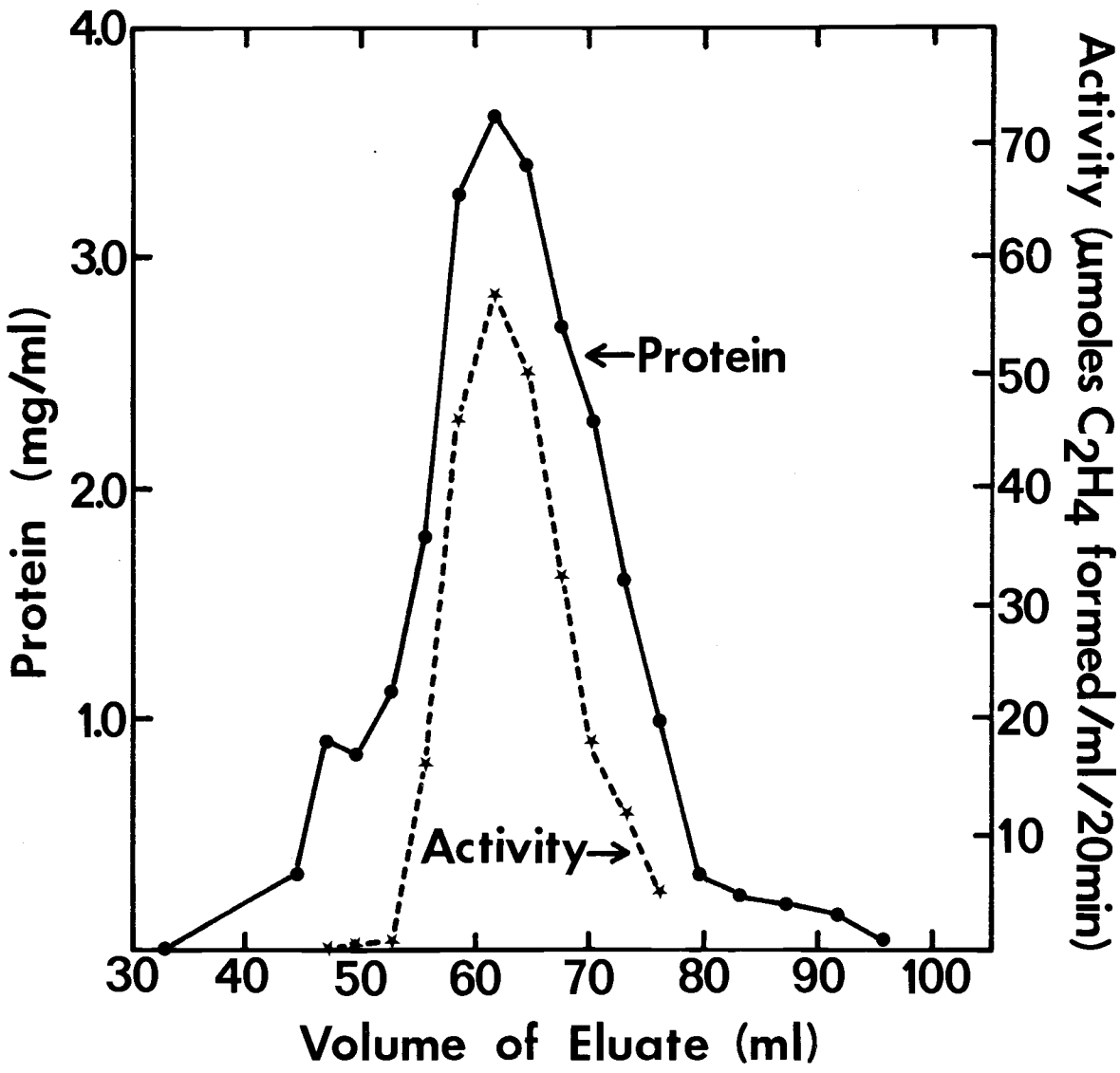


Figure 2. Chromatography of the Mo-Fe protein on a column of Sephadex G-200. The chromatographic procedure and the methods for assaying protein and C_2H_2-reducing activity are described in the Experimental Procedure section. A sample (90 mg of protein) of the partially purified Mo-Fe protein from DEAE-cellulose chromatography was placed on the column. The protein content plotted above was that determined by the method of Lowry et al. (1951). Protein corrected to dry weight may be obtained by multiplying by the factor 0.75.

and other contaminating proteins, no appreciable increase in specific activity was achieved.

RESULTS

Purity

A summary of the purification of the Mo-Fe protein from bacteroids (Table I) shows a purification of about 31-fold relative to the crude extract and that about 20% of the total Mo-Fe protein activity in the crude extract was recovered. The elution profile of the Sephadex G-200 column (Figure 2) shows that the specific activity was greatest in those fractions collected in the center of the protein peak (57-69.5 ml). In this particular experiment, these fractions catalyzed C_2H_2 reduction at a rate of 933 nmoles C_2H_4 formed per minute per mg protein. In other purification experiments, specific activities ranged from 850 to 1000.

The result of disc gel electrophoresis (Hedrick and Smith, 1968) of a sample of the Mo-Fe protein that had been treated with sodium mersalyl is presented in Figure 3 (Gel a). Over 99% of the 20 μg of protein applied to the gel migrated as one band. A close examination of the gel reveals a trace of minor components. Whether or not these are a result of the relatively harsh conditions during the electrophoresis experiment has not been established. Treatment of the protein with 1% 2-mercaptoethanol and 1% sodium dodecylsulfate followed by electrophoresis in 0.1% sodium dodecylsulfate gels (Weber and Osborn, 1969) produced one band (Figure 3, Gel b).

Table I. A summary of the purification of the bacteroid Mo-Fe protein. Activity was determined by the C_2H_2-reduction assay in the presence of an optimum amount (0.28 mg) of Fe protein from DEAE-cellulose (specific activity (C_2H_2) 500). Added Fe protein was not included in calculation of specific activity.

	Preparation	Total protein (mg)	Specific activity (units/mg)	Total activity (units)[a]	Purification factor	Recovery (%)
I	Crude extract	4700	30	141,000	1.0	100.0
II	21-41% PPG ppt, solubilized and heat treated at 55° for 2.5 min	833	90	75,020	3.0	53.7
III	DEAE-cellulose, chromatography. 161-169 ml fraction from 0.15 M NaCl eluate	86	448	38,534	14.9	27.5
IV	Sephadex G-200 chromatography. 57-69.5 ml eluate	31[b]	933	29,913	31.1	20.7

[a] A unit is defined as one nmole C_2H_4 formed from C_2H_2 per min.

[b] Protein content determined by the Lowry method was corrected to a dry weight basis (see Experimental Procedure section).

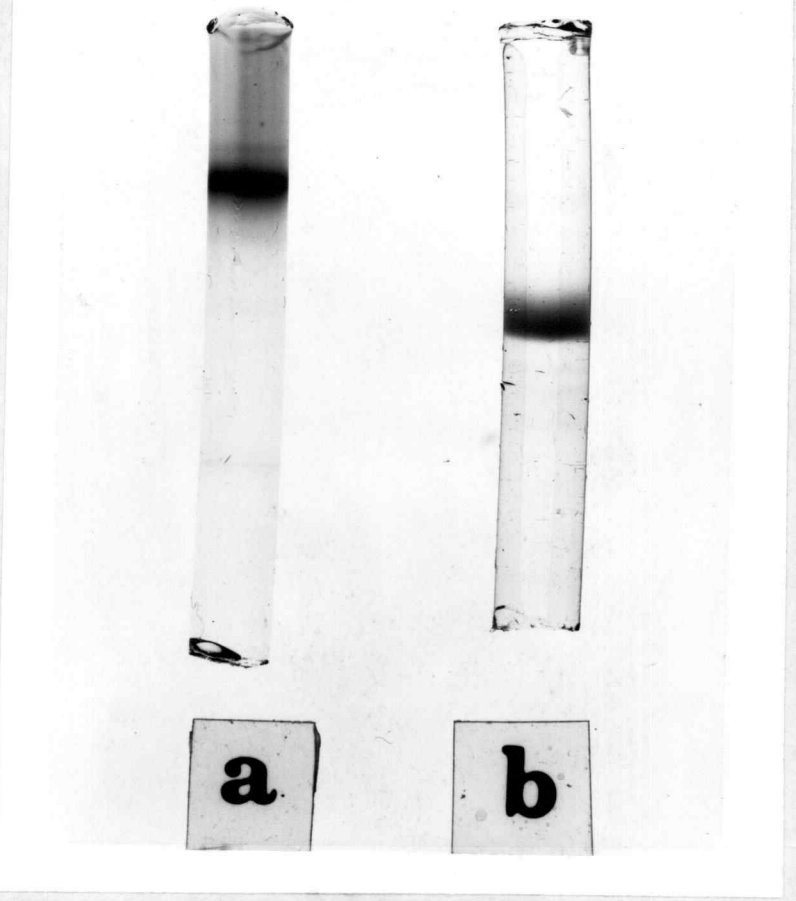


Figure 3. Electrophoretic patterns of the purified Mo-Fe protein. Gel a represents the result of electrophoresis of 20 μg of sodium mersalyl treated protein in 6% polyacrylamide gels at pH 7.9 (Hedrick and Smith, 1968). Gel b represents the result of electrophoresis of 20 μg of the purified protein in the single gel sodium dodecylsulfate system of Weber and Osborn (1969). Gels were stained with Napthol Blue Black.

From the results of these experiments it seems clear that the protein has been highly purified. This conclusion is confirmed in the sedimentation equilibrium experiments to be discussed subsequently. In some instances, Mo-Fe protein used for study of properties was purified on Biogel (A-0.5 m) columns instead of Sephadex G-200 columns. All Mo-Fe protein samples used for determining properties had specific activities (C_2H_2) that ranged from 850 to 1000 and a high degree of purity as judged by electrophoresis.

Molybdenum, Iron, Acid-labile Sulfide and Amino Acid Composition

The Mo, Fe, and acid-labile sulfide content of the Mo-Fe protein is presented in Table II. The protein contains an average of 1.3 Mo, 28.8 Fe, 26.2 acid-labile sulfide atoms based on a molecular weight of 200,000 (see "Molecular Weight and Subunit Composition"). The Mo-Fe protein contains 23 cysteic acid residues and all the common amino acids (Table III).

Partial Specific Volume

The partial specific volume ($\bar{v}$) of 0.732 ml per g for the purified Mo-Fe protein was calculated from the amino acid composition as described by Cohn and Edsall (1943).

Table II. Metal and acid-labile sulfide content of the purified bacteroid Mo-Fe protein. Analyses were made by methods described in the Experimental Procedure section and values are expressed on the basis of a Mo-Fe protein molecular weight of 200,000. Data presented are means of at least 3 replicate determinations.

Component	g-atoms per mole protein[a]
Iron	28.8±0.53
Molybdenum	1.3±0.15
Acid-labile sulfide	26.2±0

[a]Values are followed by the standard error of the mean.

Table III. Amino acid composition of the purified bacteroid Mo-Fe protein.[a]

Amino Acid	Residues/200,000 mol. wt.[b]	Amino Acid	Residues/200,000 mol. wt.[b]
Cysteic Acid[c]	23.24 (23)	Glycine	170.89 (171)
Histidine	53.37 (53)	Alanine	162.50 (162)
Arginine	93.84 (94)	Valine[e]	125.92 (126)
Lysine	122.31 (122)	Methionine	39.28 (39)
Aspartic Acid	181.98 (182)	Isoleucine[e]	111.82 (112)
Threonine[d]	89.04 (89)	Leucine	135.81 (136)
Serine[d]	109.13 (109)	Phenylalanine	80.06 (80)
Glutamic Acid	179.28 (179)	Tryptophan[f]	28.84 (29)
Proline	89.04 (89)	Tyrosine[d]	64.76 (65)

[a]Except as otherwise noted the results are the averages obtained from 20, 44 and 72-hour hydrolysates.

[b]Numbers in parentheses are nearest whole numbers.

[c]Determined on 20-hour hydrolysate (Moore, 1963).

[d]These values were obtained by extrapolating to zero time.

[e]Average of 44 and 72-hour hydrolysates.

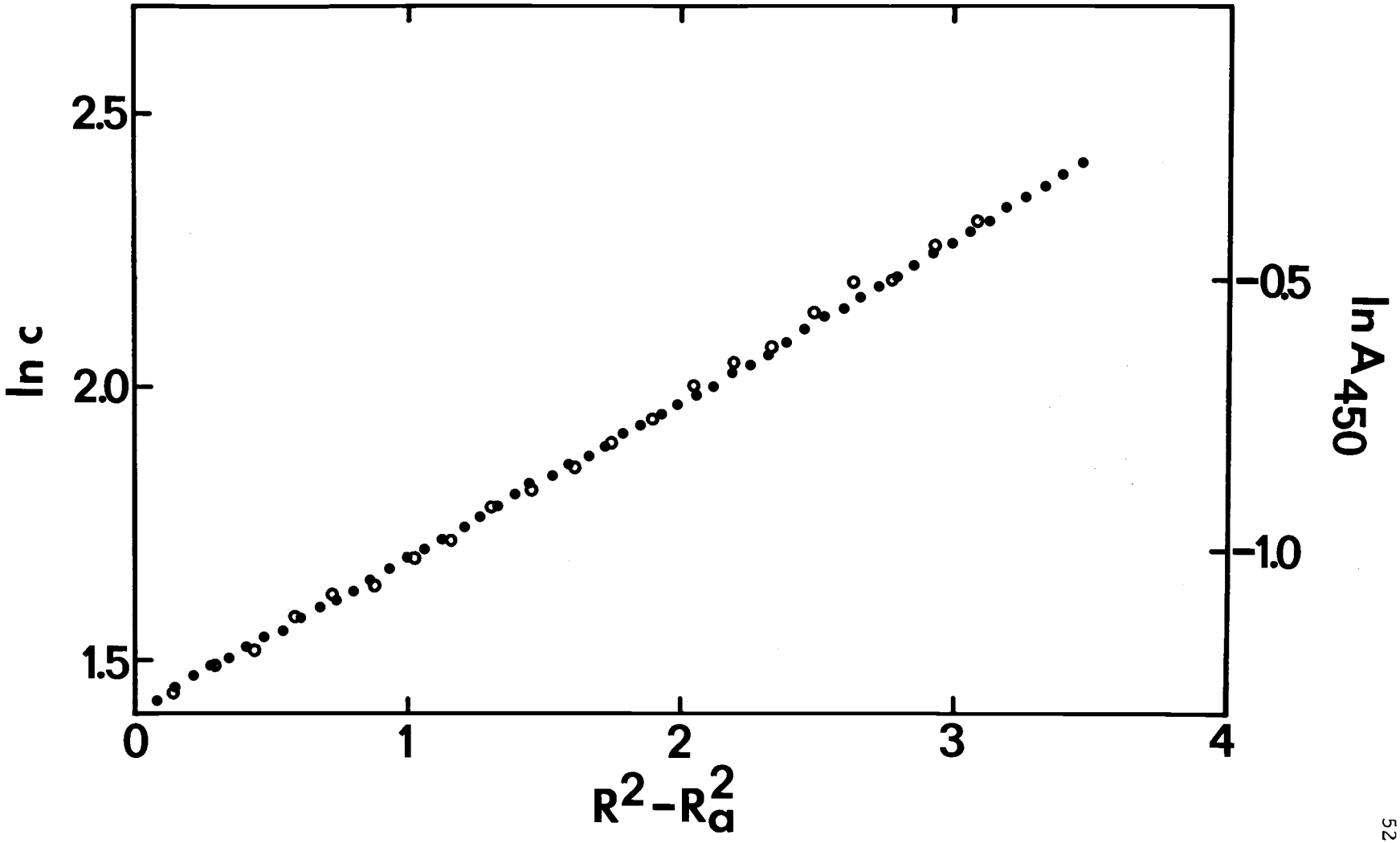
[f]Determined by the method of Edelhoch (1967).

Molecular Weight and Subunit Composition

Figure 4 illustrates the results obtained when purified Mo-Fe protein in 0.02 M TES, pH 7.5, containing 0.25 M NaCl, 1 mM dithiothreitol, 40 mM dithionite was centrifuged to equilibrium at 4883 rpm and 3.0° C (low speed experiment). The figure includes data obtained with both Rayleigh interference and scanner (A_{450}) optics. Calculations from both sets of data reveal a weight average molecular weight (M_w) of 197,600. The results of the high speed experiment are shown in Figure 5 as a plot of $M_{w,c}$ versus concentration for the three separate channels of the Yphantis (1964) centerpiece. Above 0.5 mg of protein per ml (ca. 2 fringes) the data from the three channels indicate the same molecular weight. The M_w calculated from these data is 202,000.

The molecular weight of the Mo-Fe protein also was determined in 50 mM potassium phosphate, pH 7.0, containing 6 M guanidine hydrochloride and 5 mM dithiothreitol. Plots of ln c versus $R^2 - R_a^2$ (not shown) were apparently linear. Analysis of the data revealed the nonideality expected in concentrated guanidine hydrochloride solutions. This was indicated by the positive slopes obtained when the reciprocal apparent molecular weights ($1/M^a$) were plotted against concentration (not shown) (Munk and Cox, 1972). To largely eliminate the effect of nonideality the quantity, $2 M^a_{n,c} - M^a_{w,c}$, where $M^a_{n,c}$ equals the apparent number average molecular weight and

Figure 4. Results obtained from low speed equilibrium sedimentation of the purified Mo-Fe protein. The data are plotted as the natural logarithm of the net fringe displacement, ln $\underline{c}$ (solid circles) or A_{450} (open circles) as a function of $R^2 - R_a^2$ where R_a is the radial position of the meniscus and R is the radial position of the indicated point. The initial protein concentration was 1.09 mg per ml in 0.02 M TES, pH 7.5, containing 0.25 M NaCl, 1 mM dithiothreitol, 40 mM dithionite. The rotor speed was 4883 rpm and the temperature 3.0° C.


2.5
2.0
1.5
ln c
0
1
2
3
4
$R^2-R_a^2$
-0.5
-1.0
ln A_{450}

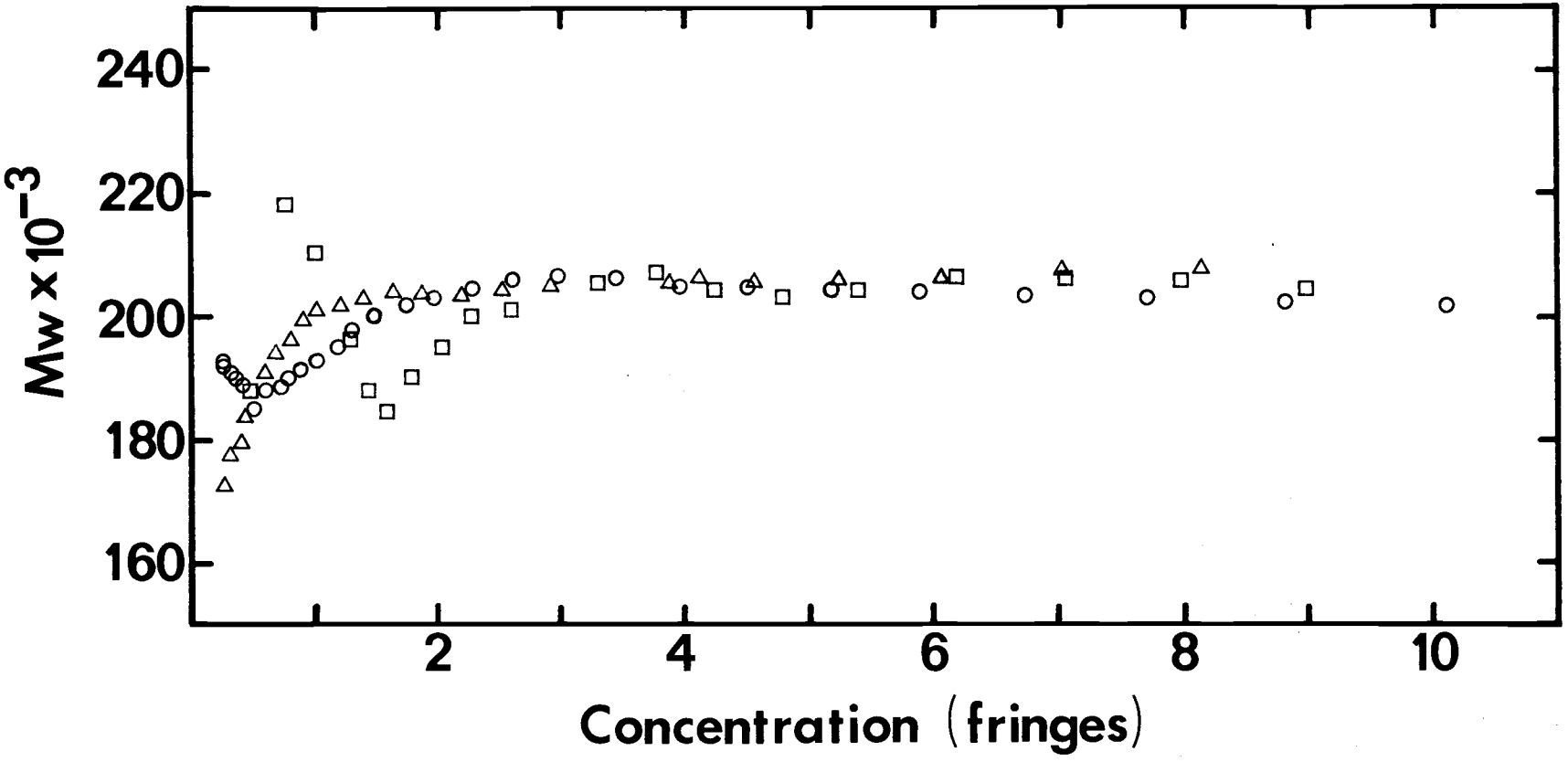


Figure 5. Molecular weight distribution obtained from high speed equilibrium sedimentation of the Mo-Fe protein. The weight average molecular weight (M_w) calculated for various concentrations is plotted as a function of the concentration. The initial protein concentration and solvent used were as described in Figure 4. The rotor speed was 13,078 rpm at 4.2° C. A Yphantis (1964) type centerpiece was used and the results from the three channels (△ outer, ○ middle, □ inner) are presented.

$M^{a}_{\underline{w, c}}$ the apparent weight average molecular weight, evaluated at the specified concentrations, was plotted against concentration in fringe displacements (Yphantis, 1964) (Figure 6). At each point, virial coefficients for $M^{a}_{\underline{n, c}}$ and $M^{a}_{\underline{w, c}}$ should cancel, otherwise upward curvature would be seen. If the protein sample is heterogenous, downward curvature would be apparent. The results (Figure 6) indicate that, within the accuracy of the measurements, the subunits in 6 M guanidine hydrochloride are homogeneous.

Most proteins that have been studied show a 0.01 to 0.012 ml per g decrease in the apparent partial specific volume ($\bar{v}^{*}$) when experiments in dilute salt solutions are compared with those in 6 M guanidine hydrochloride (Kawahara and Tanford, 1966 and Reisler and Eisenberg, 1969). A value of 0.722 for $\bar{v}^{*}$ has been used for calculation of $M^{a}_{\underline{n, c}}$ and $M^{a}_{\underline{w, c}}$. The value for $(2M_{\underline{n}} - M_{\underline{w}})_{\underline{c}=o}$ is about 47,000 g per mole ($\bar{v}^{*}$=0.722). The value for the weight average molecular weight at infinite protein dilution ($M_{\underline{w, c}=o}$) obtained from the intercept of a $1/M^{a}_{\underline{w}}$ versus concentration plot (Munk and Cox, 1972) is 47,200. If $\bar{v}^{*}$ equals 0.732, then the $M_{\underline{w, c}=o}$ is 50,300.

The purified Mo-Fe protein migrated as one band in sodium dodecylsulfate gels (Weber and Osborn, 1969) (Figure 3, Gel b). Gels were calibrated by electrophoresis of known protein standards in the system and by plotting their mobility relative to that of cytochrome _c_ as a function of the molecular weight (log scale) (Figure 7).

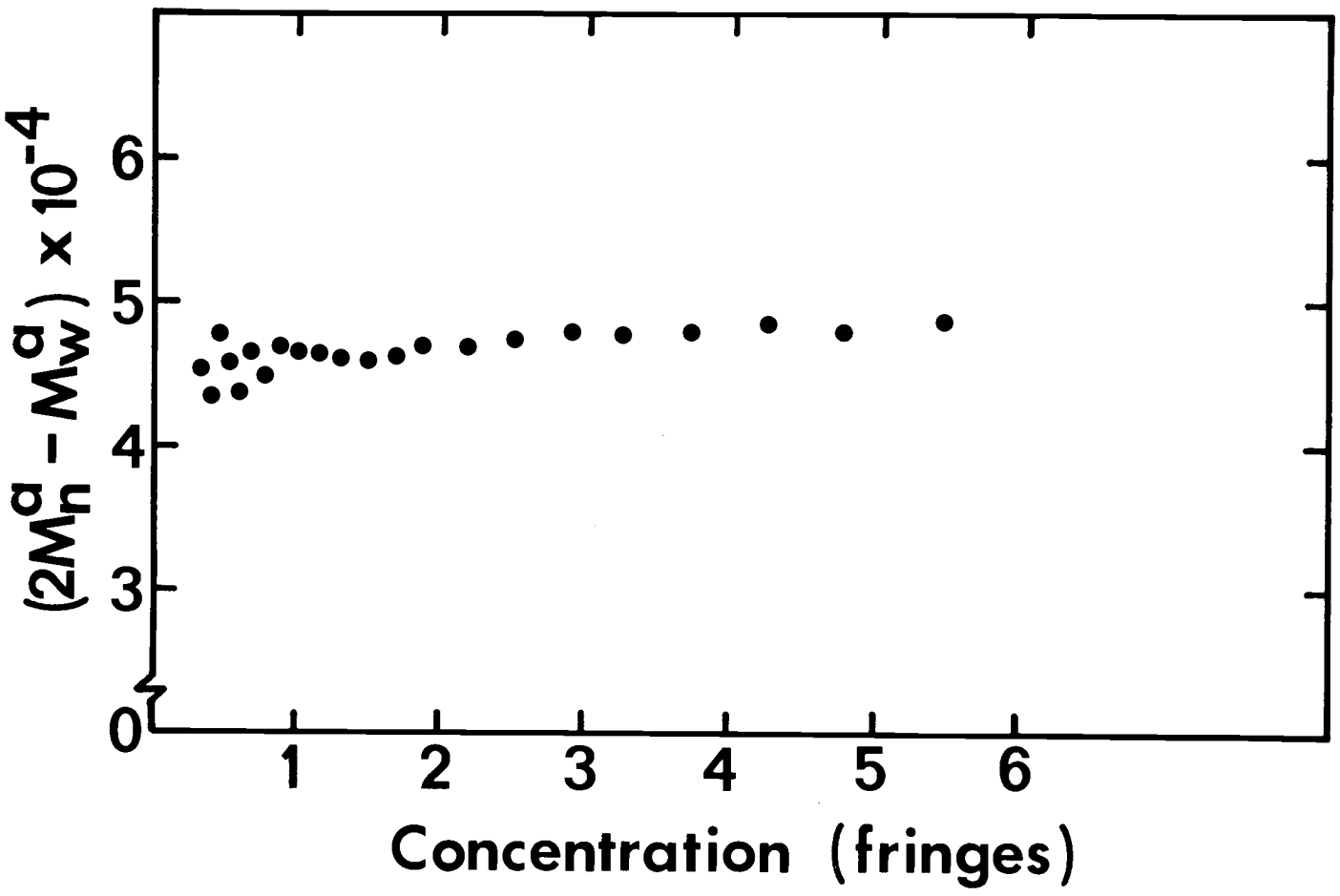


Figure 6. Apparent molecular weight distribution of the purified Mo-Fe protein in 6 M guanidine hydrochloride. Mo-Fe protein, 0.35 mg per ml in 50 mM potassium phosphate, pH 7.0, containing 6 M guanidine hydrochloride, and 5 mM dithiothreitol, was centrifuged to equilibrium at 32, 204 rpm at 19.1 °C.

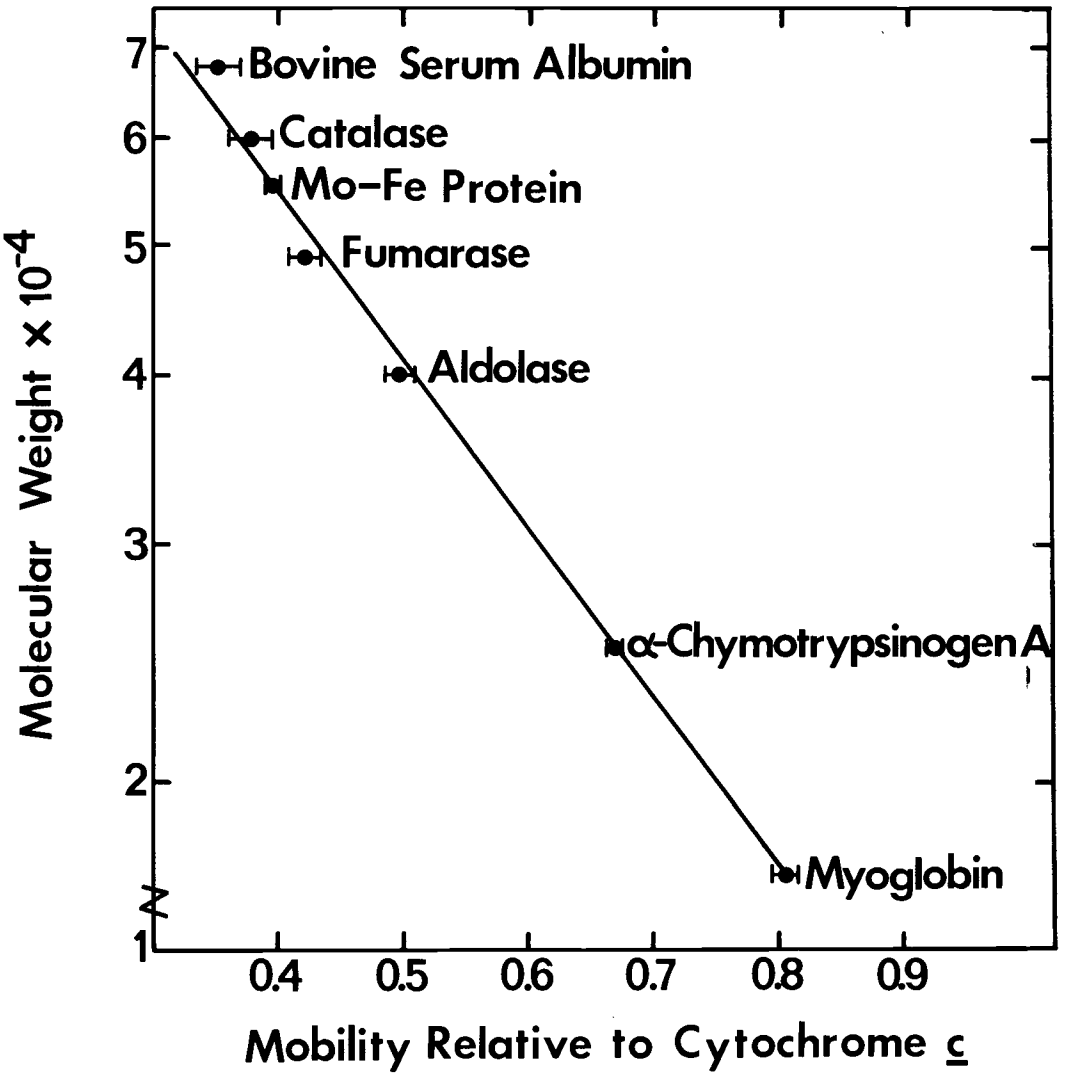


Figure 7. Subunit molecular weight of the purified Mo-Fe protein as determined by sodium dodecylsulfate gel electrophoresis. Molecular weights of the protein standards given by Weber and Osborn (1969) are: bovine serum albumin (68, 000), catalase (60, 000), fumarase (49, 000), aldolase (40, 000), α-chymotrypsinogen A (25, 200) and myoglobin (17, 200). Electrophoresis of mixtures of standard proteins (10 μg of each protein) was performed in 3 gels and the Mo-Fe protein in 7 gels. Cytochrome c was included in all samples. The mobility of each protein was measured relative to that of cytochrome c. The brackets about each point represent the standard error of the mean of the relative mobilities of each protein. The line was located by a linear-least squares fit of the data.

The calibration line (Figure 7) was located by a linear least squares fit of the data. The mobility of the Mo-Fe protein was 0.396, and from this a molecular weight of the subunit of 55,000 was obtained.

Sedimentation Coefficient, Diffusion Coefficient and Frictional Ratio

The sedimentation velocity coefficient of the Mo-Fe protein was determined at 5° C at three protein concentrations (Figure 8). From these values, a $\underline{s}^{o}_{20,w}$ of 9.99 is obtained. A diffusion coefficient ($D^{o}_{20,w}$) of 4.44×10^{-7} cm^2 s^{-1} was calculated from the $s^{o}_{20,w}$ and a frictional ratio (f/f_{min}) of 1.24 from the $D^{o}_{20,w}$ using equations given by Tanford (1961).

Absorption Spectra

The ultraviolet and visible absorption spectra of the Mo-Fe protein are presented in Figure 9. The ultraviolet spectrum shows a typical absorption maximum at 279 nm. The $\underline{E}_{279}$, based on a molecular weight of 200,000 was 3.69×10^{5} cm^{-1} M^{-1}. In the visible spectrum there is a broad absorbance in the region between 350 nm and 600 nm but no distinct shoulders are apparent. Addition of $Na_2S_2O_4$ caused a slight decrease in absorbance in the visible region. The ratio A_{280}/A_{260} is 1.3, however, the absorbance measurements for the determination of this ratio involved the use of a reference cuvette containing a Biogel eluate that was obtained just prior to the

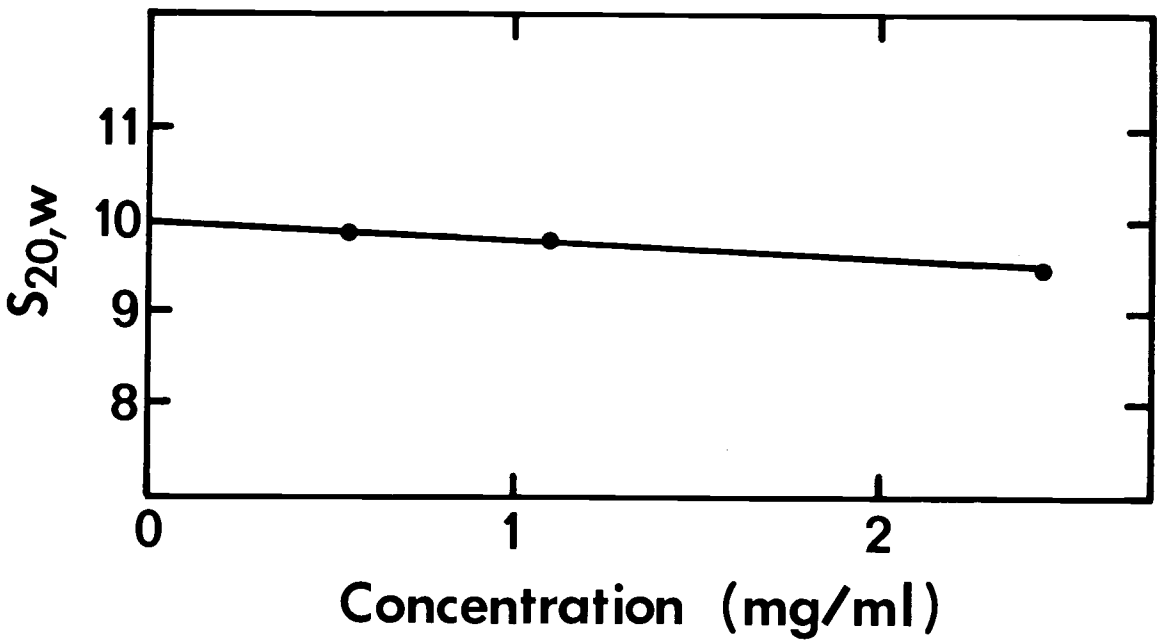
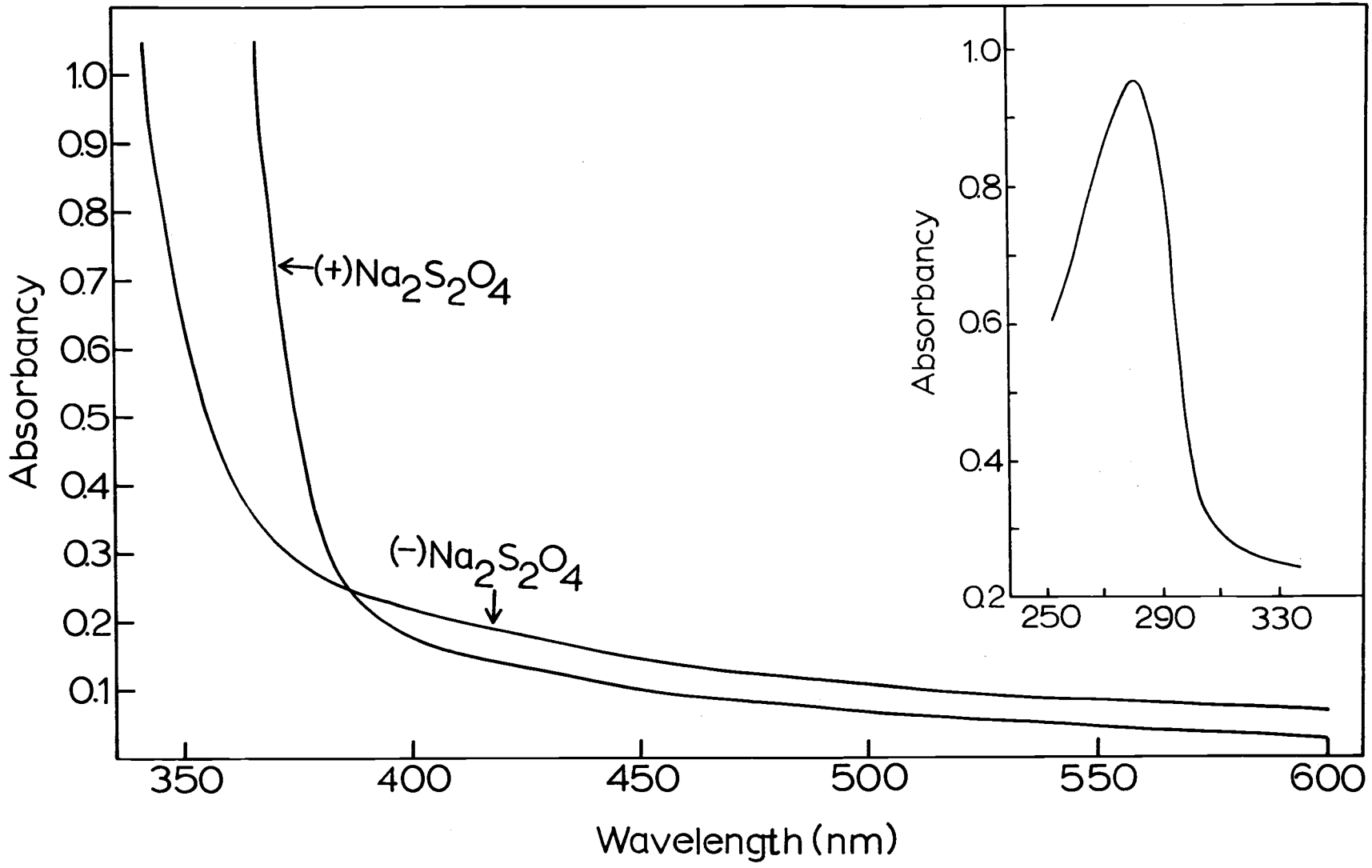


Figure 8. Effect of protein concentration on the $\underline{s}_{20,w}$ of the purified Mo-Fe protein. Mo-Fe protein at the concentrations indicated in 0.02 M TES, pH 7.5, containing 0.25 M NaCl, 1 mM dithiothreitol, and 40 mM dithionite was centrifuged at 48,096 rpm at 5.4° C. The concentration distribution (A_{280}) was recorded at selected intervals. Each sedimentation coefficient obtained was corrected to water at 20° ($\underline{s}_{20,w}$) and plotted as a function of protein concentration. The solid line is the linear-least squares fit to the data.

Figure 9. The visible and ultraviolet absorption spectra of the purified Mo-Fe protein. The protein concentration of the sample used for determination of the visible spectrum was 0.58 mg per ml. The spectrum was recorded against a reference cuvette containing 0.02 M TES, pH 7.5, 0.25 M NaCl, 1 mM dithiothreitol. The trace labeled (+$Na_2S_2O_4$) was recorded five minutes after addition of 20 μmoles of $Na_2S_2O_4$ to the sample. The protein concentration used for determination of the ultraviolet spectrum (insert) was 0.54 mg per ml. The reference cuvette and sample were prepared as described in the Experimental Procedure section.


Absorbancy
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
←(+)Na2S2O4
(−)Na2S2O4
350
400
450
500
550
600
Wavelength (nm)
Absorbancy
1.0
0.8
0.6
0.4
0.2
250
290
330

elution of the Mo-Fe protein. (See Experimental Procedure section.) It was presumed that this corrected for any absorbance due to $Na_2S_2O_4$ in the protein sample. Some error might have been involved in this method but it was not possible to maintain active, soluble Mo-Fe protein without use of $Na_2S_2O_4$.

Stability

The inactivation of the Mo-Fe protein from soybean nodule bacteroids under 0.2 atm of O_2 at 30° C is illustrated in Figure 10. About 50% of the activity of the Mo-Fe protein was lost after an exposure for 4.5 minutes, and activity was completely lost after an exposure period of 30 minutes. When the Henry's-Law constant, K, was corrected for the effect of salt on the solubility of O_2 at 30° C,[3] a value of 3.9×10^{7} was obtained. By use of this constant and Henry's Law, the concentration of O_2 in solution was calculated to be 2.16×10^{-4} M. Upon inactivation, the color of the protein changed from greenish-brown to reddish-brown.

Storage of the purified Mo-Fe protein for 24 hours at 20° C under Ar resulted in a loss of 50% of the C_2H_2-reducing activity when compared with a control sample that had been stored in liquid N_2. The purified protein may be stored in liquid N_2 for months without appreciable loss of activity.

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[3]International Critical Tables, Volume III, p. 272.

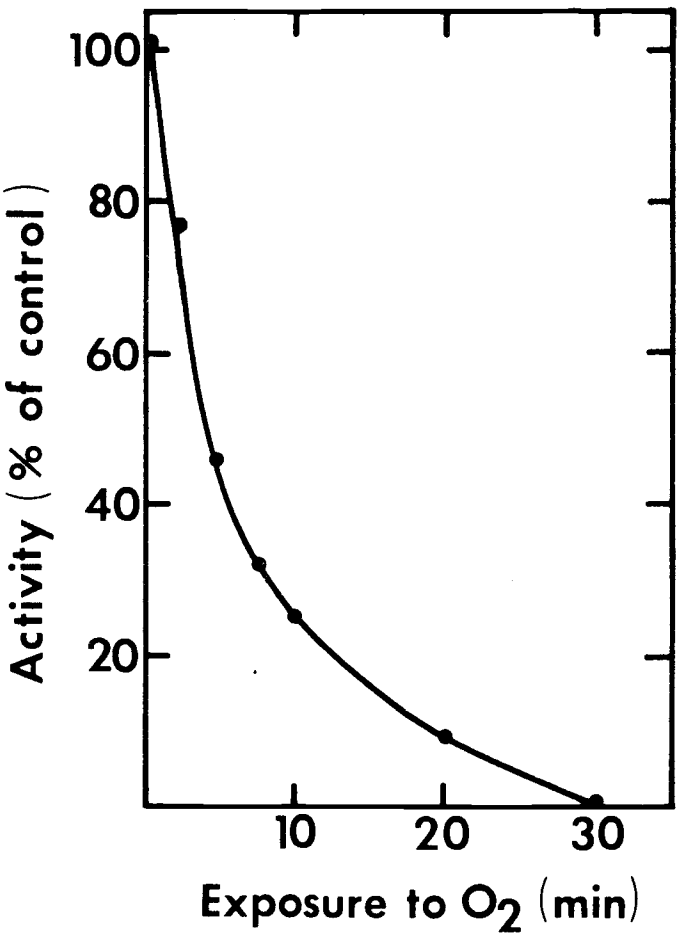

Figure 10. Inactivation of the purified Mo-Fe protein by O_2 as a function of time. Excess $Na_2S_2O_4$ was removed from the Mo-Fe protein sample by passage over a 1.5 × 25 cm column of Biogel P-30 equilibrated anaerobically with 0.02 M TES, pH 7.5 containing 0.25 M NaCl, 1 mM dithiothreitol and 0.06 mM $Na_2S_2O_4$. A sample (1.0 ml, 0.5 mg of protein per ml) was incubated in a water bath at 30° C under 0.2 atm of O_2 with shaking. At the times indicated duplicate samples were removed and assayed for C_2H_2-reducing activity as described in the Experimental Procedure section. The Mo-Fe protein (1.0 ml, 0.5 mg protein per ml) used as the control was incubated for 30 minutes at 30° C under Ar with shaking.

Titration of the Mo-Fe Protein with the Fe Protein

The restoration of N_2-fixing activity that occurs upon recombining increasing amounts of Fe protein with a fixed amount of purified Mo-Fe protein is illustrated in Figure 11. In this experiment, the addition of 312 μg of the Fe protein to 54 μg of Mo-Fe protein resulted in a maximum rate of N_2 reduction by the recombined fractions. The specific activity (N_2) of the Mo-Fe protein with an optimum level of Fe protein was 260. Addition of as much as 520 μg Fe protein caused no apparent inhibition of the Mo-Fe protein activity.

K_m for N_2

A modified Eadie-Hofstee plot of N_2 fixation as a function of pN_2 is shown in Figure 12. The data were obtained by recombining the Mo-Fe protein with an optimum level of the Fe protein. Under these conditions, the rate of electron transfer to the Mo-Fe protein should not be limiting. An apparent K_m of 0.068 atm for N_2 was obtained from these data. The solubility of N_2 in water at 30° C and 1 atm is 5.7×10^{-4} M (Hwang and Burris, 1972). Without correcting for the effect of reactants on the solubility of N_2, the K_m for N_2 on a molar basis is 3.88×10^{-5}.

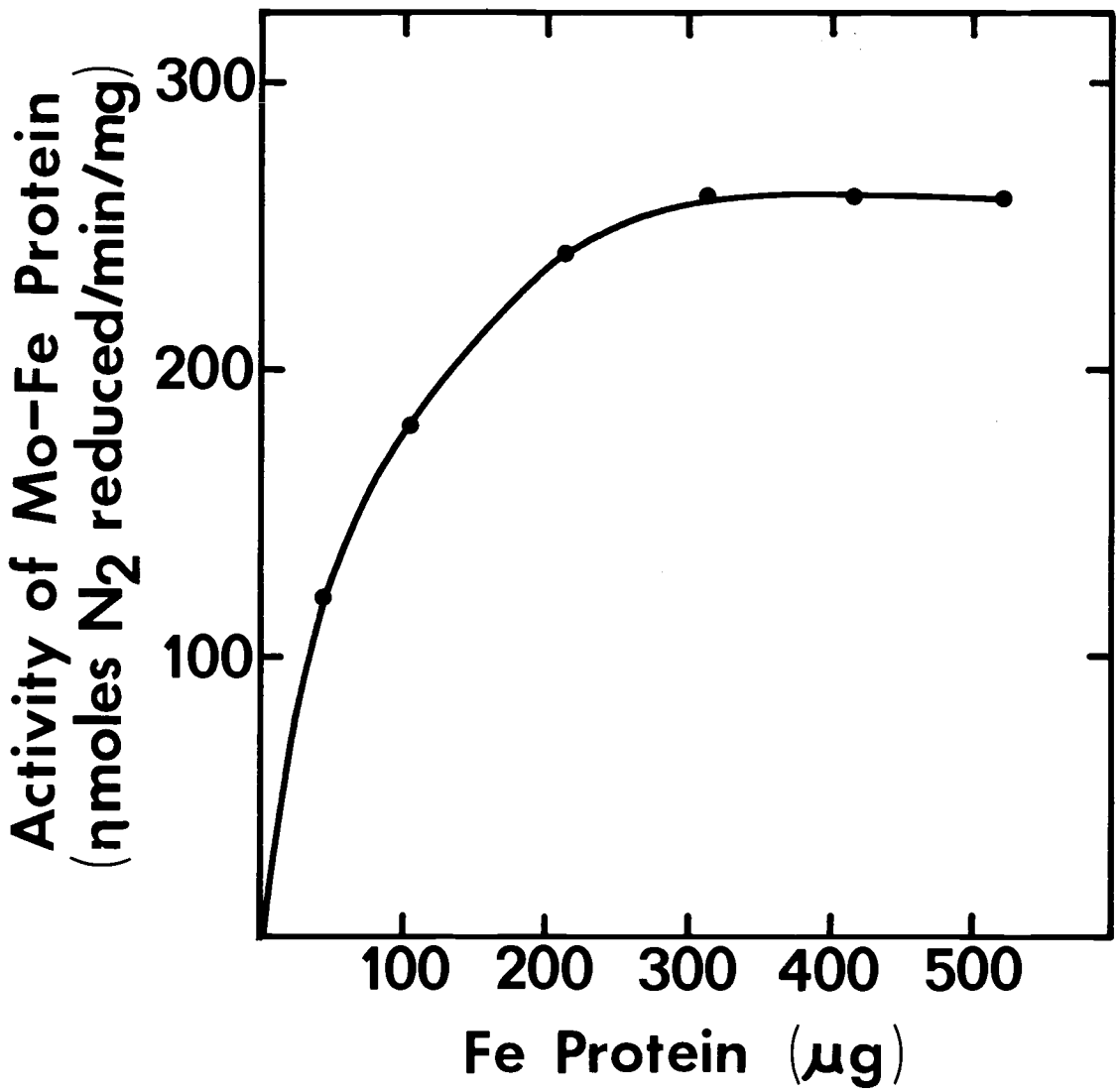

Figure 11. Titration of the purified Mo-Fe protein with the Fe protein. Each reaction mixture contained 54 μg of the purified Mo-Fe protein and the indicated amount of Fe protein (specific activity (N_2) 220). N_2-reducing activity was measured by the NH_3-synthesis assay (Experimental Procedure). Each point is the average of duplicate determinations.

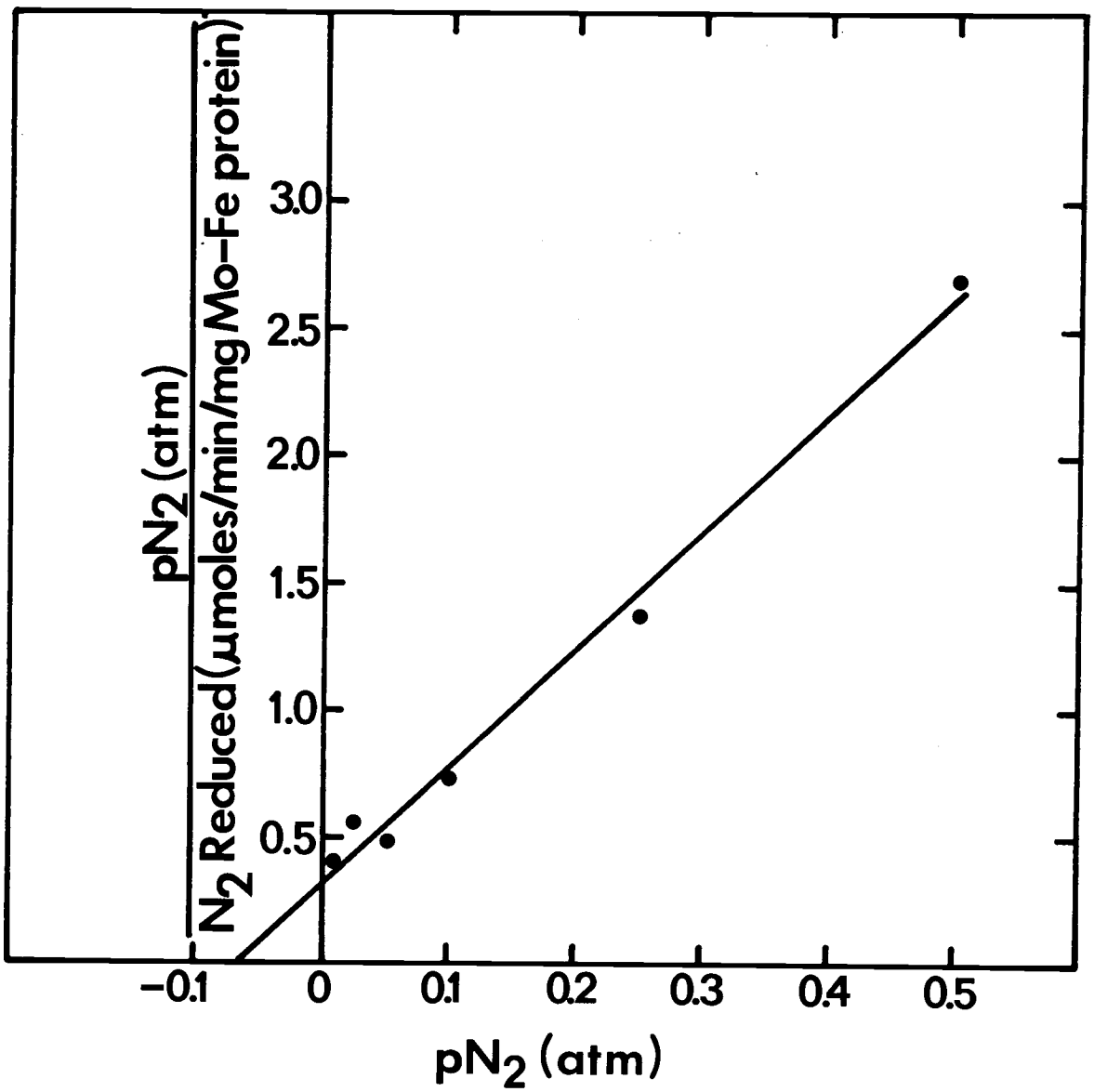

Figure 12. A modified Eadie-Hofstee plot of N_2 fixation rate as a function of pN_2. Each reaction mixture contained 54 μg of the purified Mo-Fe protein and 312 μg of Fe protein. The indicated partial pressures of N_2 were obtained by filling the reaction bottles containing all reactants except enzyme and $Na_2S_2O_4$ with 1.0 atm of Ar. Appropriate aliquots of Ar were then removed and replaced with N_2 by use of hypodermic syringes. Assays were performed as described in the Experimental Procedure section. Each point is the average of duplicate determinations. The line was located by a linear-least squares fit of the data.

DISCUSSION

Activity of the Mo-Fe Protein

The purified Mo-Fe protein from bacteroids catalyzed the reduction of N_2 and C_2H_2 only in the presence of Fe protein. This activity is dependent upon a source of ATP, Mg^{2+}, and a reductant ($Na_2S_2O_4$). Partially purified nitrogenase from soybean nodule bacteroids previously (Koch, Evans and Russell, 1967b) has been shown to catalyze ATP-dependent hydrogen evolution and the reduction of alternate substrates such as cyanide, acetylene and azide. The catalytic properties of the Mo-Fe protein from bacteroids are essentially the same as those for the purified Mo-Fe proteins from Azotobacter vinelandii (Burns, Holsten and Hardy, 1970), Klebsiella pneumoniae (Eady et al., 1972) and Clostridium pasteurianum (Vandecasteele and Burris, 1970).

The specific activity (C_2H_2) of the homogeneous Mo-Fe protein from soybean nodule bacteroids was about 900. This value is about 35% of the specific activity reported for the homogeneous Mo-Fe protein component of nitrogenase from Clostridium pasteurianum (Zumft et al., 1972, Tso, Ljones and Burris, 1972 and Huang, Zumft and Mortenson, 1973) and 75% of the value reported for the Mo-Fe protein from Klebsiella pneumoniae (Eady et al., 1972). It is possible that the catalytic capabilities of purified nitrogenase

components from different sources vary and that the bacteroid enzyme from nodules is less active than the enzyme from other sources. A more plausible explanation is that loss of Mo occurred during the purification procedure resulting in the formation of a Mo-deficient and inactive Mo-Fe protein that co-purified with the active Mo-Fe protein. Recently, Zumft et al. (1972) have reported that homogeneous Mo-Fe protein of Clostridium pasteurianum from Sephadex G-200 columns could be separated into an inactive component and an active component by chromatography on DEAE-cellulose. The inactive component lacked Mo and contained much less acid-labile sulfide and Fe than the active component. The immunoprecipitin test of this inactive protein, however, was positive against Mo-Fe protein antiserum. As a result of removing the inactive component, the specific activity (C_2H_2) of the Mo-Fe protein increased from 1200 to about 2500 and the Mo content increased to about 2 g-atoms per mole of protein (220, 000 mol. wt.). The Mo content of the bacteroid Mo-Fe protein is 1.3±0.15 g-atoms per mole of protein (200, 000 mol. wt.). It seems possible, therefore, that some Mo was lost during purification. Recently, Ganelin et al. (1972) have isolated a Mo-peptide complex (1000 mol. wt.) from the purified Mo-Fe component of nitrogenase from Azotobacter vinelandii. The Mo in the complex accounted for 50% of the Mo in the Mo-Fe protein. Loss of a Mo-peptide complex analogous to that of Ganelin et al. (1972) during purification may

explain results reported here and the results of Zumft et al. (1972).

The specific activity (N_2) of the Mo-Fe protein used in the titration experiment (Figure 11) was 260 when a saturating level of Fe protein was used. Specific activity of the same sample in the C_2H_2-reduction assay in the presence of an optimum level of Fe protein was 860. The ratio of C_2H_2 reduced to N_2 reduced is 3.3, which agrees reasonably well with the theoretical value of three. Tso, Ljones and Burris (1972) obtained a ratio of four when highly purified Mo-Fe and Fe protein components from Clostridium pasteurianum were recombined in four different ratios and assayed for the two activities.

Properties

The ultraviolet and visible absorption spectra (Figure 9) of the bacteroid Mo-Fe protein are not markedly different from the spectra reported for the Mo-Fe protein from Clostridium pasteurianum (Dalton et al., 1971) and Klebsiella pneumoniae (Eady et al., 1972). The visible spectrum (Figure 9) shows no obvious shoulder at 420 nm and no peaks at 520 nm and 550 nm upon addition of $Na_2S_2O_4$. The protein, therefore, seems to be free of cytochrome contamination. The molar extinction coefficient at 279 nm for the bacteroid Mo-Fe protein is 3.69×10^5 cm^{-1} M^{-1} as compared to the values of 3.30×10^5 cm^{-1} M^{-1} at 277.5 nm for the analogous protein from

Klebsiella pneumoniae (Eady et al., 1972), and 4.7×10^5 cm^{-1} M^{-1} at 280 nm for the analogous protein from Azotobacter vinelandii (Burns and Hardy, 1972).

The linear relationship in the plot of ln$c$, where c equals net fringe displacement or A_{450}, versus $R^2 - R_a^2$ (Figure 4) and the plot of M_w versus c (Figure 5) indicates a high degree of homogeneity for the Mo-Fe protein. The absorption spectrum of the Mo-Fe protein isolated here and the spectra of analogous proteins isolated from other organisms (Eady et al., 1972, Burns and Hardy, 1972 and Dalton et al., 1971) show considerable absorption in the visible region (350-600 nm). Rayleigh interference optics can be used to follow the sedimentation of all proteins while the sedimentation of ferredoxin-like proteins such as the Mo-Fe proteins of nitrogenases can be followed by use of absorbance at 450 nm. If the Mo-Fe protein prepared in this study was contaminated by a protein not absorbing at a wavelength of 450 nm, then a difference in the equilibrium distribution as determined by A_{450} or Rayleigh interference patterns would have been observed. The results of Figure 4 indicate that this is not the case. These results have been obtained with Mo-Fe protein which had been stored in liquid N_2, then thawed and treated with fresh $Na_2S_2O_4$ just prior to placing into the centrifuge cells. In several experiments (not presented) the addition of $Na_2S_2O_4$ was omitted and upward curvature in plots analogous to those in Figure 4 was always observed. The results

suggested aggregation under these conditions.

The results of both high and low speed equilibrium experiments indicate an average M_w of 199, 800 for the native Mo-Fe protein. The $M_{w, c=o}$ of 47, 200 to 50, 300 obtained for the protein in 6 M guanidine hydrochloride and the homogeneous nature of the subunits (see Results section) argues strongly that the native protein contains four subunits of equal molecular weight. Further evidence for the presence of only one size of subunit in the Mo-Fe protein from bacteroids is the result of disc gel electrophoresis in sodium dodecylsulfate (Figure 3, Gel b) where only one protein band was observed. Although the molecular weight of 55, 000 determined by the electrophoretic method (Figure 7) is higher than that determined by sedimentation equilibrium experiments, it has been established that the accuracy of the electrophoretic method in the molecular weight range between 50, 000 and 200, 000 is ±10% (Weber and Osborn, 1969). If the assumption is made that the estimate of 55, 000 is 10% too high, the molecular weight of the subunit determined by the two methods is in fair agreement. The finding of one size of subunit represents a major difference between the Mo-Fe protein from bacteroids and the analogous Mo-Fe proteins from Klebsiella pneumoniae (Eady et al., 1972), Clostridium pasteurianum (Dalton et al., 1971 and Huang, Zumft, and Mortenson, 1973) and Azotobacter vinelandii (Burns and Hardy, 1972). The Mo-Fe proteins isolated from the latter free-living, N_2-fixing

microorganisms have two sizes of subunits.

The Mo-Fe protein component of nitrogenase from bacteroids was 50% inactivated when exposed to 0.2 atm of O_2 at 30° C for 4.5 minutes and totally inactivated within 30 minutes. Under similar conditions, 50% inactivation of the Mo-Fe protein from Klebsiella pneumoniae required 10 minutes and total inactivation required more than 60 minutes (Eady et al., 1972). Dalton et al. (1971) also reported that a 60 minute exposure of the Mo-Fe protein from Clostridium pasteurianum to air was required for total inactivation. The Mo-Fe protein from bacteroids also lost 50% of its catalytic activity when stored at 20° C for 24 hours. Under similar conditions, the analogous protein from Klebsiella maintained full catalytic activity for 50 hours (Eady et al., 1972). Thus, the Mo-Fe protein from bacteroids is considerably more sensitive to O_2 than the analogous proteins from Klebsiella and Clostridium. Also, the protein from bacteroids is more sensitive to heat than the analogous protein from Klebsiella.

The Mo-Fe protein from bacteroids has some properties that are similar to properties of Mo-Fe proteins from other sources (Burns, Holsten and Hardy, 1970, Eady et al, 1972 and Dalton et al., 1971). Although it has a somewhat higher Fe and acid-labile sulfide content than other Mo-Fe proteins, it, like other Mo-Fe proteins, contains between 1 and 2 g-atoms of Mo per mole of protein and approximate equivalences of Fe, acid-labile sulfide and cysteic acid

(cysteine and cystine). The Mo-Fe protein from bacteroids, like other Mo-Fe proteins, is acidic as evidenced by the fact that it is tenaciously bound to DEAE-cellulose. The amino acid composition of the bacteroid Mo-Fe protein is not strikingly different from analogous proteins from Klebsiella pneumoniae (Eady et al., 1972) or Azotobacter vinelandii (Burns and Hardy, 1972). Estimation of the homology (Harris and Teller, 1973) of Mo-Fe proteins from these three sources has resulted in divergence (D) values of less than 0.04 for each pairing and, therefore, suggests a high degree of homology.

It will be of interest to determine whether nitrogenase components from nodules of a variety of leguminous species are consistently different from comparable components from free-living N_2-fixing bacteria.

SUMMARY

This investigation was conducted to develop a procedure for the purification of the Mo-Fe protein component of nitrogenase from bacteroids of soybean nodules and to determine some of the chemical and physical properties of the Mo-Fe protein. The results of this investigation may be summarized as follows:

1. The procedure for the purification of the Mo-Fe protein included a polypropylene glycol fractionation and heat treatment of crude extracts, followed by chromatography on DEAE-cellulose and Sephadex G-200. The Mo-Fe protein purified by this procedure had a high degree of homogeneity as determined by disc gel electrophoresis and sedimentation equilibrium experiments.
2. The specific activities of several preparations ranged between 850 and 1000 for C_2H_2 and 260 and 300 for N_2. A ratio of C_2H_2 reduced to N_2 reduced of 3.3 and an apparent K_m for N_2 of 0.068 atm were obtained when the Mo-Fe protein was assayed in the presence of an optimum level of Fe protein.
3. An average molecular weight of 199,800 was determined for the protein by low speed sedimentation equilibrium methods and by the high speed sedimentation equilibrium method. Sedimentation equilibrium analysis of the protein in 6 M guanidine hydrochloride revealed one size of subunit with a molecular weight of

about 50,000. Treatment of the protein with sodium dodecylsulfate and 2-mercaptoethanol followed by electrophoresis in gels containing sodium dodecylsulfate also produced one size of subunit. From these results, it was concluded that the Mo-Fe protein from bacteroids is a tetramer composed of four subunits of equal size.

4. Ultracentrifugal analysis of the protein revealed a $\underline{s}^{o}_{20,w}$ value of 9.99, and a partial specific volume ($\bar{v}$) of 0.732 ml per g was calculated for the protein from the amino acid composition. A $D^{o}_{20,w}$ of 4.44×10^{-7} cm^2 s^{-1} was calculated from the $\underline{s}^{o}_{20,w}$ and a frictional ratio (f/f_{min}) of 1.24 from the $D^{o}_{20,w}$.
5. Analyses of samples of the protein revealed a mean of 1.3 Mo, 28.8 Fe and 26.2 acid-labile sulfide atoms per molecule, based on a molecular weight of 200,000 for the protein. The protein contained all of the common amino acids.
6. The time for 50% inactivation of the protein under 0.2 atm of O_2 at 30° C was 4.5 minutes. The protein lost 50% of its catalytic activity when stored at 20° C for 24 hours under argon.
7. The ultraviolet spectrum of the protein exhibited an absorbance maximum at 279 nm and a molar extinction coefficient of 3.69×10^{5} cm^{-1} M^{-1} at this wavelength. A broad general absorbance was observed in the visible spectrum between 350 and 600 nm, and no obvious shoulders or peaks were apparent.

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