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THE EFFECTS OF Mg^{2+} , ATP AND ADP ON ATP HYDROLYSIS AND ELECTRON
TRANSFER BY AZOTOBACTER VINELANDII NITROGENASE

by

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I. INTRODUCTION

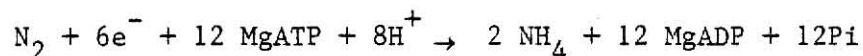
Biological nitrogen-fixation can be performed by a minority of the procaryotes including bacteria and blue-green algae, but the process it catalyzes is, needless to say, essential for life on earth. The research in this field is quite in a vigorous growth since the world's food and energy supply is in a crucial period. Industrial nitrogen fertilizers are energy costly, but the organisms involved use solar-energy and optimize the amount of ammonia necessary for the field.

A. Nitrogen-Fixing Free-Living Bacteria [2]

Among the nitrogen-fixing bacteria, the so-called free-living types are able to fix nitrogen without the cooperation of other organisms. Such well-known free-living type bacteria are the Azobacteriaceae, many of the anaerobic saccharolytic clostridia and many blue-green algae of the *Anaebaena* and *Nostoc* genera. The free-living heterotrophic nitrogen-fixing bacteria may be classified into three categories: (a) Obligatory aerobes, (b) Facultative anaerobes, and (3) Strict Anaerobes. The group of obligatory aerobes consists mainly of the members of Azotobacteraceae. Bacteria of Azotobacteraceae, with the exception of members of the genus *Beijerinckia*, are large organisms which show their most regular and constant cell size and shape during the exponential growth phase when supplied with a suitable carbon and energy source and nitrogen source.

B. Physiology of Nitrogen-Fixing Bacteria [2]

Although there exists a wide variation in type of nitrogen-fixing organisms, the central reaction according to which biological nitrogen-fixation proceeds and the enzymes responsible for this reaction are the same in all of these organisms.



From this reaction, it can be seen that nitrogen fixation, in addition to reducing power (6 electrons per one nitrogen molecule to ammonia), requires energy to proceed. Although there is not complete agreement, it is usually assumed that 12 moles of ATP are required for one mole of nitrogen reduced to two moles of ammonia. In free-living nitrogen-fixing organisms, the electrons derived from degradation of various types of carbon compounds flow to ferredoxin, an electron carrier of low redox potential, and subsequently via nitrogenase reduce dinitrogen to ammonia. In addition to this reaction, the ATP-requiring nitrogenase system may catalyze a number of other reductions such as 2 protons to 1 dihydrogen molecule, acetylene to ethylene, azide to dinitrogen + ammonia, cyanide to methane + ammonia, et cetera.

Since acetylene reduction to ethylene can easily be estimated by gas chromatography, acetylene reduction is generally used as a rapid method for measuring nitrogen fixation both in vitro and in vivo. In nitrogenase-containing cell-free extracts from nitrogen-fixing bacteria, any electron donor able to reduce ferredoxin (or some other suitable electron carrier) in the presence of ATP or an ATP-generating system is able to reduce dinitrogen to ammonia, acetylene to ethylene, etc. The usual way of studying nitrogen-fixation by cell-free extracts is to use sodium dithionite as the electron donor. In the presence of ATP, this compound directly reduces nitrogenase and hence dinitrogen or an alternative substrate without ferredoxin. The enzyme mechanisms will be discussed further in section E.

C. Nitrogenase and Its Structure

Nitrogenase is comprised of two proteins: Fe protein and MoFe protein.

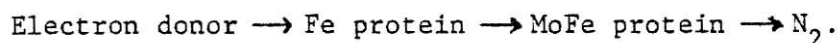
The nitrogenase proteins must interact during catalysis and form a complex. Because the complex dissociates during each catalytic cycle, the Fe protein is called component 2 and MoFe protein is called component 1; and, especially for nitrogenase system of Azotobacter vinelandii, component 1 is denoted by Av 1 and component 2, Av 2 respectively. Despite their necessity of affinity for each other, the two proteins can be completely separated, and they can be obtained free of other proteins by standard techniques. This is also true for the nitrogenase proteins of Azotobacter vinelandii, even though much work has been done with a particulate form of the nitrogenase of this organism. The purification of active nitrogenase proteins is straightforward except for one feature: both proteins, Av 1 and Av 2, are rapidly inactivated by oxygen, and they must always be handled under strict anaerobic conditions. The Av1 has molecular weight around 216,000 (Kleiner and Chen) [28] and consists of 4 subunits; these are of two different types. They contain 20-30 iron atoms, 20-30 acid-labile sulfur atoms, and 2 Mo per MoFe protein molecule of 4 subunits. The Fe protein is the smaller of the two proteins, and its molecular weight is around 56,000 (Kleiner and Chen) [28]. It consists of two identical subunits. Present consensus favors one Fe_4S_4 cluster per Fe protein molecule of two subunits. It is further speculated that the Fe_4S_4 cluster bridges two identical and symmetrically combined subunits, being anchored through two cysteines on each subunit.

Electron donors can be ferredoxin, flavodoxins or dithionite ion. ATP and divalent metal ion, usually Mg^{2+} , are absolute requirements for any electron transfer reactions by nitrogenase; the observed number of ATP molecules consumed is often higher, but never lower than indicated. Besides dinitrogen, a number of other molecules and ions can be reduced through

nitrogenase catalysis, e.g., N_2O , N_3^- , C_2H_2 , HCN , CH_3NC , H^+ .

The reduction of acetylene to ethylene is of great importance in practical measurement of nitrogenase activity in vivo, and the reduction of protons to dihydrogen is important because this substrate is always present with active nitrogenase systems. In fact, total electron transfer is as fast under argon (with H^+ as only reducible substrate) as under N_2 , and N_2 reduction under 1 atm N_2 is accompanied by some H_2 evolution. Hence, the rate of activation and reduction of dinitrogen is not limiting for the earlier steps of electron transfer and energy coupling.

Our present view of electron transfer sequence in nitrogenase is:



This fits well with the popular but unproven supposition that molybdenum is part of the N_2 -reducing site. The Fe protein shows a ferredoxin type EPR signal in its reduced site, and this signal disappears when the system of both nitrogenase proteins is allowed to exhaust the supply of reductant (dithionite). The MoFe protein exists in three EPR-defined oxidation states: an oxidized state, which is EPR-silent, is formed by dye-oxidation and is probably not part of the catalytic cycle; the native state, which is observed in the presence of dithionite and shows the characteristic EPR spectrum; and the super-reduced state, which is also EPR-silent and is formed only through reduction by the Fe protein in the presence of ATP. The super-reduced state returns to the native state upon exhaustion of dithionite.

D. Binding of ATP to the Fe Protein [1]

Bui and Mortenson used gel filtration to show that ATP binds to the

Fe protein, but not to the MoFe protein. Tso and Burris extended equilibrium binding studies to quantitative experiments with a gel equilibrium technique. They found that ATP binds to two independent and equivalent sites on the Fe protein from C. Pasteurium with a specific dissociation constant, $K_s = 17$ micro M. ADP competes for binding at one site for ATP. The dissociation constant of ADP is 5 micro M [3,4].

Mg^{2+} (or certain other divalent metal ions) is necessary for any enzymic activity of nitrogenase. The metal ions react with ATP to form an ATP-Mg complex, and it is this complex that reacts with Fe proteins; and, the same applies to ADP as inhibitor [5].

Zumft et al. titrated the Fe protein from C. Pasteurium with ATP using EPR and found that 2 ATP per Fe protein was necessary for complete conversion of the EPR spectrum from a rhombic to an axial type [6-9]. A newer hypothesis originating from the EPR shift is that the binding of ATP to the Fe protein leads to a conformational change which alters the environment of the Fe_4S_4 cluster and allows for energy coupling during electron transfer from this group to some group on the MoFe protein. Binding of ATP lowers the redox potential of the Fe protein from -0.29 V to -0.40 V. These measurements were made at equilibrium, and there is much more energy available from ATP hydrolysis in turnover. However, the potential shift is a first step in the energy transfer process which requires hydrolysis of ATP and only takes place in the complex of the component proteins [10].

The other evidence for interaction between the ATP sites and the iron-sulfur site on the Fe protein was provided by Walker and Mortenson [11]. They discovered that ATP dramatically increases the rate of reaction between native Fe protein and the iron chelator a, a'-dipyridyl; Mg^{2+} is required

for this effect, ADP inhibits, and other nucleotides have little or no effect. The MoFe protein does not show such response to ATP. ATP binding to the Fe protein probably exposes the Fe_4S_4 cluster to solvent. Kinetic studies by T. Ljones and R. H. Burris also support the evidence that both of the two ATP sites must be occupied to allow the conformational change that increases the reactivity of the iron-sulfur site [15].

ATP-binding to the Fe protein probably exposes the Fe_4S_4 cluster to solvent, a conclusion drawn for the following reasons:

1. The EPR shift with ATP is similar to the shift with 5 M urea, a denaturant that most likely exposes the iron-sulfur site [12].
2. The increased rate of chelation seems consistent with increased exposure as ferredoxins increase their chelation rates upon denaturation.[13].
3. The decrease in the redox potential is a shift in the expected direction when the Fe_4S_4 cluster is moved from the interior to the exterior of the protein, judging from the trends observed with synthetic iron-sulfur model compounds.[14].

E. Stoichiometry and Kinetics of ATP Hydrolysis

The number of ATP molecules hydrolyzed per electron transferred (ATP/e^-) during nitrogenase catalysis is variable. Values of 10 or more have been observed with no apparent upper limit, but a lower limit of 2 is indicated. The implication is that ATP hydrolysis can be uncoupled from electron transfer, but electron transfer must be coupled with ATP hydrolysis and that the stoichiometry during tight coupling is 2 ATP/e^- . This stoichiometry is also supported by the binding and kinetic studies with ATP, which are consistent with ATP hydrolysis at two sites coupled with the transfer of one electron from the single iron-sulfur site on the Fe protein [4, 15, 16].

However, kinetic studies of ATP utilization by nitrogenase are compli-

cated by the rapid onset of product inhibition by ADP [17,18]. Early claims for complicated, sigmoid kinetics at high ATP levels were based on experiments where uncontrolled product inhibition prevented the measurement of true initial velocities. More careful measurement of initial velocities, in the presence of an ATP-generating system, revealed no obvious deviation from hyperbolic behavior in the range of ATP concentration from the apparent Michaelis constant (0.1 - 0.4 mM) [5,17]. Nevertheless, at lower ATP levels, the relationship becomes clearly sigmoid[19]. It is expected if electron transfer from the Fe protein requires ATP hydrolysis at both of the two sites. ADP blocks one of these sites and reveals the sigmoid curve of the plots at higher level of ATP concentration. The inhibition by ADP provides a mechanism for regulation of nitrogen fixation, and this seems appropriate for such an energy-costly process. There is no need, however, to postulate special regulatory sites for ADP [4, 15].

F. Bioenergetic Aspects of ATP Hydrolysis [20]

The Gibbs free energy of formation of NH_4^+ from dinitrogen and H_2 is negative. The energy of ATP hydrolysis is, therefore, not needed to drive an otherwise unfavorable equilibrium in the desired direction, but rather to overcome the high activation energy barrier for cleavage of stable nitrogen-nitrogen bond. Nitrogenase most certainly lowers the activation energy for dinitrogen reduction, but the remaining barrier is still high that the reaction will only proceed with the input of extra energy. T. Ljones argued for a model of two ATP being hydrolyzed while one electron is transferred from the Fe_4S_4 cluster on the Fe protein onto the MoFe protein. Further speculation suggests that energy is conserved at this step by the reduction of a group on the MoFe protein with a lower redox potential than that of the

donor group on the Fe protein. Thus, the energy released in ATP hydrolysis could be used for driving the electrons to a low potential, and subsequently these highly reactive reduced groups would be able to overcome the activation energy barrier for N_2 reduction. Accordingly, nitrogenase carries out reductive dephosphorylation. A calculation from the standard Gibbs free energy at pH 7 for hydrolysis to ADP suggests that 2 ATP/e^- lowers the potential energy by 0.75 V. as the electrons are entering the system at approximately the level of the hydrogen electrode at pH 7, or somewhat lower. This means that with reasonably complete conservation of energy at this step, groups on the MoFe protein with mid-point potentials of -1.0 V or lower are reduced. No evidence on such low potentials have been provided so far, but several lines of circumstantial evidence are consistent with the proposal [21].

Other functions for ATP in the nitrogenase system have been proposed. Some of these proposals are alternatives to reductive dephosphorylation; some are both. Hageman and Burris [29] showed that the complex dissociates after each electron is transferred between the component proteins, and in their studies of the mechanism of inhibition of electron transfer by excess MoFe protein, they proved that the major route of electron transfer is via free, oxidized Fe protein. Emerich et al. [22] investigated the properties of the inactive complex between the Fe protein of C.pasteurium and the MoFe of A.vinelandii and discovered that the two ATP sites on the Fe protein in the complex are accessible for binding of ATP; but, the Fe_4S_4 cluster is not accessible to reaction with chelators in the presence of ATP. With one of these proteins as inhibitor of another homologous nitrogenase, substrate reduction is inhibited completely, but ATP hydrolysis is inhibited

partially. The heterologous complex is much tighter than the homologous complexes [22].

Thus, the following model is consistent both with these observations and those of Smith et al. [30]. The normal sequence of events is that the free Fe protein binds two molecules of ATP and accepts one electron, followed by binding to the MoFe protein, electron transfer, and ATP hydrolysis. Dissociation of the complex is required before the Fe protein accepts another electron [23].

From the experimental observations and discussions so far, it seemed necessary to study Mg ion effect, ATP concentration effect, and ADP concentration effect on the nitrogenase system from Azotobacter vinelandii as a model. The studies done in this paper are designed especially in view of kinetics of nitrogenase to determine the effectors of nitrogenase system. The observations and data obtained in this study are discussed.

II. MATERIALS AND METHODS

A. Chemical Methods

a. Ammonia Assay [2]

The amount of ammonia reduced from dinitrogen by nitrogenase was measured by a somewhat simple microdiffusion apparatus and successive measurement of absorbance with the Chaykin reagent. Reactions with nitrogenase preparations are run directly in the vaccine bottle stoppered with a vaccine stopper. The reaction is terminated by adding 1 ml of saturated potassium carbonate, and the vaccine stopper is replaced with a stopper carrying a glass rod (fig. 1) whose tip has been dipped in 5 N sulfuric acid, touched to a paper towel and immediately inserted into the bottle. The bottle is placed on a desk overnight to diffuse ammonia to the acid on the glass rod. After diffusion is completed, the glass rod is transferred immediately to a vaccine bottle containing Chaykin reagent and shaken twice. After 15 minutes for complete color development, absorbance is measured at 625 nm by Beckman UV spectrophotometer.

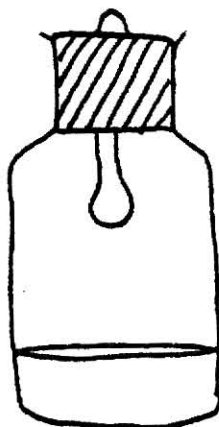


Figure-1

Reagents for ammonia assay: [2]

1 mM $(\text{NH}_4)_2\text{SO}_4$

0.14 M Phenol solution

2.5 mM Sodiumnitro ferricyanide

0.75 M NaOH

6% Sodium hypochlorite

Chaykin reagent:

Reagent A: 37.5 ml of 0.14 M phenol solution and 1 ml of nitro-prusside solution

Reagent B: 10 ml of 0.75 NaOH solution and 0.2 mls of 6% Sodium hypochlorite solution.

Standard Curve:

The following amounts of the 1 mM ammonium sulfate were put into single reaction vials.

<u>Vial #</u>	<u>micro liter $(\text{NH}_4)_2\text{SO}_4$</u>	<u>micro liter H_2O</u>	<u>nmole NH_4</u>
1	25	125	25
1-a	25	125	25
2	50	100	50
3	75	75	75
4	100	50	100
5	150	0	150
6	0	150	-
6-a	0	150	-

The order of addition of each reagents must be: (1) H_2O , (2) $(\text{NH}_4)_2\text{SO}_4$, and then reagent A, reagent B successively. The readings of absorbance was

done at 625 nm, after having been allowed to diffuse overnight, using a Beckman UV spectrophotometer.

b. Acetylene Reduction Assay [2].

Acetylene is reduced to ethylene by nitrogenase and gives the most quantitative analysis among possible nitrogen activity assays so far developed. The acetylene, and the ethylene produced during nitrogenase reaction, are measured by gas chromatography with a relatively sensitive hydrogen flame ionization detector. Routinely, acetylene produced from calcium carbide and water is used, which has adequate purity for acetylene-ethylene assay. The acetylene gas-generating device is illustrated in fig. 2. The lower vessel part is filled completely with distilled water, and a small amount of water is also present in the upper part of the vessel. The serum stopper is removed from the sidearm of the lower vessel, a few pellets of calcium carbide are dropped in, and sidearm is stoppered immediately. A needle is inserted through the serum stopper to withdraw acetylene, after the acetylene evolution is completed.

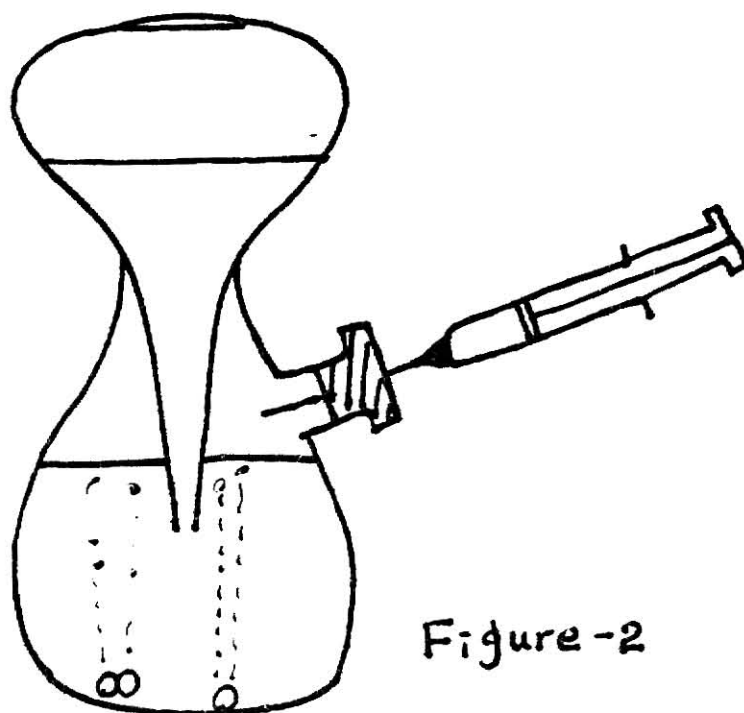


Figure -2

Standard Peak for Acetylene and Ethylene Assays:

1 ml of ethylene and 1ml of acetylene were injected into a 125 mls vaccine bottle stopped with a rubber stopper. The amount of acetylene and ethylene in this bottle are calculated as follows:

$$(1 \text{ ml}/24,000 \text{ mliters/mole}) * (1\text{ml}/125 \text{ ml}) = 33333 * 10^{-9} \text{ moles/ml.}$$

Therefore, if 300 μ l of the bottle content is injected, it must give 100 nmoles of both acetylene and ethylene. To get standard peak, 300 micro liter of the bottle content was injected into Varian Aerograph 1200 to give 40 - 50 squares for each peak of acetylene and ethylene at attenuator 256 and sensitivity 10^{-11} . The column temperature was at 65°C , injector temperature at 38°C , and detector temperature at 130°C . The carrier gas was nitrogen and the detector was hydrogen flame ionization detector.

After the nitrogenase assay, .3 ml of gaseous phase was extracted by an appropriate syringe and injected into GLC as soon as possible to keep all sample gases. The ethylene peak and acetylene peaks are counted by their squares and converted to nmoles per sample as shown below.

$$\left[\frac{\text{Squares of sample}}{\text{Squares of std.}} \times \frac{\text{attenuator of sample}}{\text{attenuator of std.}} \times \frac{\text{sensitivity of sample}}{\text{sensitivity of std.}} \times 100 \times \frac{.30}{.30} \times \frac{5.3}{.3} \right] \text{ gives nmoles per sample bottle.}$$

c. Phosphate Assay [24]

Purification and in vitro study of various enzymes which form inorganic phosphate as a product of the enzyme reaction often requires the rapid determination of inorganic phosphate in the presence of labile phosphate esters and various enzyme substrates. The desired characteristics of such an assay are: minimal hydrolysis of labile phosphate esters, lack of inter-

ference from components of the enzyme-assay system, ease and speed of performance, and sensitivity of phosphate esters under the very acidic conditions. The Fiske and Subba Row method [31] was minimized by the extraction procedure of Berenblum and Chain [32] and subsequent modifications by others. However, such an extraction procedure is not practical for the large number of samples processed daily during purification and study of the properties of an enzyme. The degree of hydrolysis of labile esters was also reduced by Lowry and Lopez [33] by performing the determination at pH4. However, for our purposes, this is unsatisfactory because of the lack of sensitivity, interference by sulfides, and excessive ATP breakdown by high levels of molybdate. The enzymic method, while of interest to be studied, has three additional enzymes involved, each of which has its own set of optimum conditions. Sufficient sensitivity for our purposes was achieved by the use of stannous chloride as suggested by Kuttner and Cohen [34]. Interference by the sulfhydryl group was avoided by addition of formaldehyde and the pH was adjusted to above 4 with acetate buffer. The resulting method had proved very satisfactory for study of an ouabain-inhibited ATP-ase in Dr. W. B. Rathbun's laboratory.

Procedure for Phosphate Assay -

Reagents:

2% ammonium molybdate (0.50 grams in 25 ml of H_2O)

3 M acetate buffer (1:1 3 N NaAc and 3 M AcOH mixture)

37% formaldehyde solution

6.75 mM $SnCl_2$ (38.1 mg stannous chloride, 0.1 ml glacial AcOH
and diluted to 25 mls with water)

1 mM K_2HPO_4 (114 mg/500 ml) for phosphate source for standard curve

3% CCl_3COOH (Quenching agent for nitrogenase reaction)

At the appropriate time, the enzyme assay solution was mixed with ice-cold TCA solution and cooled in an ice bath for approximately 15 minutes. Then, develop color by adding 0.1 ml of ammonium molybdate along with one surface of the bottle wall without stirring. Successively, add 0.2 mls of stannous chloride solution and mix well to develop color well. The reading of absorbance was done after exactly 15 minutes color development by a Beckman spectrophotometer at 725 nm. The standard curve was obtained by making the following standard curve assay protocol.

Standard Curve:

<u>Vial #</u>	<u>K_2HPO_4 solution</u>
1	0 micro liter
2	5 micro liter
3	10 micro liter
4	15 micro liter
5	20 micro liter
6	25 micro liter

Note: Each standard vial contains 1.45mls of TCA solution, 1.1 mls of acetate and formaldehyde mixture (10:1) and distilled water to make a total volume of 2.55 mls for all standard vials. (It is possible to ignore adding water to equalize.

d. Hydrogen Evolution Assay [2]

H₂ evolution during nitrogenase reaction was assayed by an ordinary thermal conductivity gas chromatography (Modified Carle 8501).

In enzyme studies (b) through (e), 500 micro liters of gaseous phase in the reaction vials were taken out by an appropriate injector after the reaction vials were quenched by a 30% TCA solution.

The gaseous content was then injected into a 1.83 m columns of Porapak S at 30°C, and the nitrogen carrier gas was set at a flow rate of 13 ml./min.

B. Enzyme Studies

a. Mg²⁺ Effect on Nitrogenase Activity [18]

This experiment was designed to determine how different levels of Mg²⁺ concentration affect nitrogenase activity. The measurement for nitrogenase activity was done by the rate of hydrolysis of ATP to ADP and Pi, as the amount of Pi can be measured spectrophotometrically with Chaykin's reagent. Ethylene reduced from acetylene and ammonia from di-nitrogen are also detectable by corresponding assay methods described in the Chemical Methods section.

Reagents:

The following reagents were freshly prepared and placed in each reaction vial. (The total volume of reaction vial was around 6.2 ml).

(1) ATP stock solution

This ATP stock solution is a mixture of 0.75 mls of 0.1 M MgAc, 8.25 mls of KHEPES buffer at pH 8.1, 48 mg of disodium salt of ATP and adjusted to pH 7.5 with ca. 20 micro liters of 5N NaOH solution.

(2) Sodium dithionite solution

A 105 mg of sodium dithionite was dissolved in 6 mls of 0.013 N NaOH solution under anaerobic condition; i.e., before dissolving sodium dithionite, the bottle must be stoppered with a rubber stopper and evacuated to be replaced with argon gas or nitrogen gas under atmospheric pressure.

(3) 0.1 M MgAc solution in KHEPES buffer

(4) Acetylene gas

A 0.5 mls of newly prepared acetylene gas was injected into each

reaction vial.

(5) Enzyme solution

This contains component 2 and component 1 at a molar ratio of 2.5:1.0.

Procedure:

The following reagents were placed in reaction vials under anaerobic conditions (either N_2 or argon) and placed in water-bath at $26^\circ C$ in experiments a-1 through a-3.

<u>RUN</u>	<u>ATP solution (ml)</u>	<u>H₂O (ml)</u>	<u>0.1 M MgAc (ml)</u>	<u>Na Dithionite Solution (ml)</u>	<u>Enzyme Solution (ml)</u>
1	0.6	0.1	0.0	0.1	0.2
2	0.6	0.05	0.05	0.1	0.2
3	0.6	0.0	0.1	0.1	0.2

EXPERIMENT a-1: Mg^{2+} Effect on ATP Hydrolysis

As per time schedule, 0.2 ml of enzyme solution was injected into the reaction vials which were being shaken mechanically for a designated time interval (0, 2, ..., 30 minutes).

After each time interval, 100 micro liters of reaction mixture were taken out by a syringe and immediately quenched in two ice-cooled quencher bottles containing 1.1 mls of 37% formaldehyde and 3 M acetic acid mixture in 3 N sodium acetate buffer solution. The quenched reaction mixture was analyzed for phosphate assay. (Refer to Chemical Methods section).

EXPERIMENT a-2: Mg^{2+} Effect on Acetylene Reduction under Argon Atmosphere

The Mg^{2+} effect on acetylene reduction was done under the same conditions as in experiment a-1 except that all reaction vials were equilibrated

with 0.5 ml of newly prepared acetylene gas before the reaction commenced.

Upon the completion of each reaction at the designated time, the reaction was stopped by injecting 0.1 ml of 30% trichloroacetic acid solution to the reaction mixture. A 500 micro liter gaseous phase was taken out by a syringe and was injected into GLC for ethylene and acetylene assays.

EXPERIMENT a-3: Mg²⁺ Effect on Dinitrogen Reduction

The test on the effect of Mg²⁺ on dinitrogen reduction was done under N₂ atmosphere. The other reaction conditions were the same as in experiments a-1 and a-2. As soon as the reaction was completed at the designated time, 500 micro liters of reaction mixture were taken out and immediately quenched by 1.0 ml of saturated potassium carbonate solution in a vaccine bottle, and then stoppered with a specialized glass-tip stopper containing 30% sulfuric acid at its tip. The vaccine bottle was swirled gently and allowed to stand overnight for a sufficient ammonia diffusion. The collected ammonia, in the form of ammonium sulfate on the glass tip, was color-developed with Chaykin's reagent for measuring absorbance at 725 nm by Beckman spectrophotometer (Chemical Methods, section a).

b. ADP Effect on Nitrogenase Activity [25]

ADP is the product of ATP during ATP hydrolysis, and it is necessary to observe its effect on nitrogenase activity. ADP effects on ATP hydrolysis, acetylene reduction and dinitrogen reduction were examined. The final concentrations of Mg^{2+} and ATP were fixed at 10 mM and 5 mM respectively for all reaction conditions.

Reagents:

ADP stock solution

10 mM sodium salt of ADP in KHEPES buffer at pH 7.5

ATP stock solution*

Sodium dithionite solution*

Enzyme solution*

KHEPES buffer*

(*These were the same as those used in section (a) of Enzyme studies.)

EXPERIMENT b-1: ADP Effect on Acetylene Reduction

The following reagents were placed in reaction vials under argon ambient; and, 0.5 ml of freshly prepared acetylene gas was injected and equilibrated by vigorous mechanical shaking at 26°C. Each run was composed of 6 reaction vials.

<u>RUN</u>	<u>ATP Stock (ml)</u>	<u>KHEPES (ml)</u>	<u>ADP Stock (ml)</u>	<u>Na-dithio- nite (ml)</u>	<u>Enzyme (ml)</u>	<u>Acetylene (ml)</u>
1	0.6	0.10	0.00	0.2	0.1	0.5
2	0.6	0.08	0.02	0.2	0.1	0.5
3	0.6	0.06	0.04	0.2	0.1	0.5
4	0.6	0.04	0.06	0.2	0.1	0.5

As per time schedule, 0.1 ml of enzyme solution was injected into the reaction vials which were shaken mechanically. After a designated time interval of 0, 1, 2, 3, 4 and 6 minutes, 100 micro liters of reaction mixtures were taken out, and 50 micro liters were injected into two quenching bottles. The remaining reaction mixture in the reaction vial was quenched by 0.1 ml of 30% trichloroacetic acid solution. A 500 micro liter of gaseous phase was taken out and injected into GLC for acetylene reduction assay.

EXPERIMENT b-2: ADP Effect on ATP Hydrolysis, Hydrogen Evolution and Dinitrogen Reduction (Simultaneous Assay)

The following reagents were placed in reaction vials (gaseous phase was replaced by dinitrogen gas) and equilibrated at 26°C. Each run is comprised of 6 reaction vials.

<u>RUN</u>	<u>ATP Stock (ml)</u>	<u>KHEPES (ml)</u>	<u>ADP Stock (ml)</u>	<u>Na-dithio- nite (ml)</u>	<u>Enzyme (ml)</u>	<u>N₂ (atm)</u>
1	0.6	0.10	0.00	0.2	0.1	1
2	0.6	0.08	0.02	0.2	0.1	1
3	0.6	0.06	0.04	0.2	0.1	1
4	0.6	0.4	0.6	0.2	0.1	1

As per time schedule, 0.1 ml of enzyme solution was injected into the reaction vials being shaken mechanically. After a designated time interval of 0, 1, 2, 3, 4 and 6 minutes, 100 micro liters of reaction mixture were taken out, and 50 micro liters were injected into two quenching vaccine bottles for phosphate assay. Simultaneously, 500 micro liters of gaseous

phase was taken out for hydrogen evolution assay by GLC. Immediately, 500 micro liters of the remaining reaction mixture was taken out and quenched by 1.0 ml of saturated sodium bicarbonate solution for ammonia assay. (Refer to Chemical Methods section).

c. ATP Effect on Nitrogenase Activity [29]

This experiment was geared towards determining the effect of different ATP levels on nitrogenase activity, varying the ATP concentration from 0.5 mM to 5.0 mM at fixed concentration of Mg^{2+} .

Reagents:

The different levels of ATP solutions were prepared as follows:

5 mM ATP stock solution 48 mg Na-ATP, 8.25 ml of KHEPES, 0.75 ml of 0.1 M MgAc and adjusted pH at 7.5

2 mM ATP solution 2 ml of 5 mM ATP stock solution and 3 ml of 0.1 M MgAc/KHEPES mixture

1 mM ATP solution 1 ml of 5 mM ATP stock solution and 4 ml of 0.1 M MgAc/KHEPES solution mixture

0.5 mM ATP solution 0.5 ml of 5 mM ATP stock solution and 4.5 ml of 0.1 M MgAc/KHEPES solution mixture.

MgAc solution*

Enzyme solution*

Sodium dithionite solution*

(*These solutions were the same as those used in sections (a) and (b) of Enzyme studies.)

EXPERIMENT c-1: ATP Effect on ATP Hydrolysis and Hydrogen Evolution
(Simultaneous Assay)

The following reagents were placed in reaction vials under argon ambient at 26°C.

<u>RUN</u>	<u>ATP level (mM)</u>	<u>Enzyme Solution (ml)</u>	<u>Na-dithionite (ml)</u>	<u>MgAc Solution (ml)</u>
1	5.0	0.1	0.2	0.1
2	2.0	0.1	0.2	0.1
3	1.0	0.1	0.2	0.1
4	0.5	0.1	0.2	0.1

Each run is comprised of 6 reaction vials with 0.6 ml of designated ATP solution and the corresponding volume of the other reagents. A 0.5 ml of newly prepared acetylene gas were injected into each reaction vial and allowed to equilibrate at 26°C for 10 minutes with a vigorous mechanical shaking.

As per time schedule, 0.1 ml of enzyme solution was injected into the reaction vials which were mechanically being shaken during the reaction. After each time interval (0, 1, 2, 3, 4 and 6 minutes), 100 micro liters of reaction mixture were taken out, and 50 micro liters were injected in two quenching bottles containing 1.1 mls of 37% formaldehyde and 3 M acetic acid mixture in 3 N sodium acetate buffer solution for phosphate assay. Simultaneously, the remaining reaction mixture was quenched by 0.1 ml of 30% trichloroacetic acid solution, and 500 micro liters of the gaseous phase were taken out for hydrogen assay by GLC.

EXPERIMENT c-2: ATP Effect on ATP Hydrolysis, Acetylene Reduction and Dinitrogen Reduction (Simultaneous Assay)

The following reagents were placed in reaction vials under dinitrogen ambient at 26°C.

<u>RUN</u>	<u>ATP level</u> <u>(mM)</u>	<u>MgAc</u> <u>Solution (ml)</u>	<u>Enzyme</u> <u>(ml)</u>	<u>Na-dithionite</u> <u>(ml)</u>	<u>N₂</u> <u>(atm)</u>
1	5.0	0.1	0.1	0.2	1
2	2.0	0.1	0.1	0.2	1
3	1.0	0.1	0.1	0.2	1
4	0.5	0.1	0.1	0.2	1

Each run consists of 6 reaction vials with 0.6 ml of a designated ATP solution as well as the corresponding volume of the other reagents.

A 0.5 ml of newly prepared acetylene gas was injected into each reaction vial and allowed to equilibrate at 26°C for 10 minutes with vigorous mechanical shaking. After each time interval (0, 1, 2, 3, 4 and 6 minutes), 100 micro liters of reaction mixture were taken out, and 50 micro liters were injected into two quenching bottles containing 1.1 ml of 37% formaldehyde and 3 M acetic acid mixture in 3 N sodium acetate buffer solution. Simultaneously, 500 micro liters of the gaseous phase in the reaction vial were taken out for acetylene assay. Immediately, 500 micro liters of the remaining reaction mixture was taken out and quenched by 1.0 ml of saturated sodium carbonate solution for ammonia assay. (Refer to section (a) of Chemical Methods).

d. ADP/ATP Ratio Effect on Nitrogenase Activity [18]

This experiment was aimed at determining the integrated effect of ATP concentration at different levels of energy charge on ATP hydrolysis, hydrogen evolution, acetylene reduction and dinitrogen reduction.

Reagents:

ADP solution*

KHEPES solution*

HEPES solution*

Enzyme solution*

(*These solutions were the same as those used in Enzyme studies, sections (a), (b) and (c).)

The solutions of different levels of energy charge were prepared as follows:

<u>Stock Solution #</u>	<u>ATP (Na salt) (mg)</u>	<u>ADP solution (ml)</u>	<u>KHEPES (ml)</u>	<u>HEPES (ml)</u>	<u>Energy Charge</u>
1	96	0.0	1.2	16.5	1.00
2	96	0.6	0.6	16.5	0.96
3	96	1.2	0.0	16.5	0.93
4	96	1.8	0.0	15.9	0.89

EXPERIMENT d-1: ADP/ATP Ratio Effect on ATP Hydrolysis, Hydrogen Evolution and Dinitrogen Reduction (Simultaneous Assay)

0.6 ml, 0.24 ml, 0.15 ml and 0.06 ml of each ATP/ADP stock solution were placed in 4 reaction vials with an appropriate amount of the other reagents shown in the following table. A total of 16 reaction vials were

evacuated and replaced by N_2 gas. As per time schedule, 0.1 ml of enzyme solution was injected into the reaction vial and allowed to proceed the reaction for 4 minutes. After 4 minutes of reaction, 100 micro liters of reaction mixture was taken out, and 50 micro liters were quenched into two quenching vaccine bottles for phosphate assay. At the same time, 500 micro liters of the remaining reaction mixture were taken out and immediately quenched by 1.0 ml of saturated K_2CO_3 solution. The rest of the reaction mixture was then quenched by 0.1 ml of 30% trichloroacetic acid, and 500 micro liters of gaseous phase were taken out for hydrogen assay by GLC.

<u>Vial #</u>	<u>Stock (ml)</u>	<u>10 mM MgAc (ml)</u>	<u>H₂O (ml)</u>	<u>Na-dithionite (ml)</u>	<u>Enzyme Solution (ml)</u>
1	0.6	0.0	0.1	0.2	0.1
2	0.24	0.36	0.1	0.2	0.1
3	0.15	0.45	0.1	0.2	0.1
4	0.06	0.54	0.1	0.2	0.1

EXPERIMENT d-2: ADP/ATP Ratio Effect on Acetylene Reduction

The four different levels of ATP stock solutions were placed in reaction vials with the other reagents of an appropriate volume as shown below. The reaction vials were evacuated and replaced for argon gas. A 0.5 ml of acetylene gas was then injected into all reaction vials and allowed to equilibrate at 26°C for 10 minutes.

<u>RUN</u>	<u>Stock (ml)</u>	<u>10 mM MgAc (ml)</u>	<u>H₂O (ml)</u>	<u>Na-dithio- nite (ml)</u>	<u>Acetylene (ml)</u>	<u>Enzyme Solution (ml)</u>
1	0.6	0.0	0.1	0.2	0.5	0.1
2	0.24	0.36	0.1	0.2	0.5	0.1
3	0.15	0.45	0.1	0.2	0.5	0.1
4	0.06	0.54	0.1	0.2	0.5	0.1

The reaction was commenced by an injection of 0.1 ml of enzyme solution and was allowed to proceed reaction for 2 minutes. After 2 minutes of reaction, the reaction mixture was quenched by 0.1 ml of 30% trichloroacetic acid solution, and 500 micro liters of gaseous phase was taken out for ethylene/acetylene assay by GLC.

e. Ratio Effect of Component 2 to Component 1 on Nitrogenase Activity [27]

The purpose of this experiment is to determine the ratio effect of component 2 to component 1 at a higher ratio of 5.0 to 1.0 as well as the energy charge effect at different levels.

In enzyme studies (a) through (d), the ratio of component 2 to component 1 was 2.5 to 1.0 molar, while here it was 5 to 1.

Reagents:

ATP/ADP stock solution*

Sodium dithionite solution*

KHEPES solution*

HEPES solution*

Enzyme solution (ratio of component 2 to component 1 is 5.0:1.0)

(*These solutions are the same as those used in enzyme studies - section (d).)

EXPERIMENT e-1: Effect of Higher Component Ratio on ATP Hydrolysis

0.6 ml, 0.24 ml, 0.15 ml and 0.06 ml of each ATP/ADP stock solution were placed in 4 reaction vials with a corresponding amount of the other reagents as follows:

<u>RUN</u>	<u>Stock (ml)</u>	<u>10 mM MgAc (ml)</u>	<u>H₂O (ml)</u>	<u>Na-dithionite (ml)</u>	<u>Enzyme Solution (ml)</u>
1	0.6	0.0	0.1	0.2	0.1
2	0.24	0.36	0.1	0.2	0.1
3	0.15	0.45	0.1	0.2	0.1
4	0.06	0.54	0.1	0.2	0.1

A total of 16 reaction vials were evacuated and replaced by argon gas. As per time schedule, a 0.1 ml of the enzyme solution was injected into the reaction vial and was allowed to proceed the reaction for 0, 2, 4 and 6 minutes. After the designated reaction time, 100 micro liters of the reaction mixture was taken out, and every 50 micro liters was quenched in a quenching bottle for phosphate assay.

EXPERIMENT e-2: Effect of a Higher Ratio of Component 2 to Component 1
on Hydrogen Evolution

The stock solutions and the reagents were the same as those in experiment e-1. The gaseous phase of reaction vials was replaced by argon gas and placed in water bath at 26°C.

The stock solution and the reagents were set up in the same manner as those found in the table of experiment e-1. After all reaction vials were equilibrated at 26°C, 0.1 ml of enzyme solution was injected and allowed to proceed the reaction for 2 minutes. After the reaction period, the reaction was stopped by 0.1 ml of 30% trichloroacetic acid solution, and 500 micro liters of the gaseous phase was assayed by GLC for hydrogen evolution.

EXPERIMENT e-3: Effect of a Higher Ratio of Component 2 to Component 1
on Acetylene Reduction Assay

The stock solutions and the reagents were the same as those used in experiment e-1, but all reaction vials were injected with 0.5 mls of acetylene gas after the gaseous phase had been replaced by argon gas. The stock solution and the reagents were set up as follows:

<u>RUN</u>	<u>Stock (ml)</u>	<u>10 mM MgAc (ml)</u>	<u>H₂O (ml)</u>	<u>Na-dithio- nite (ml)</u>	<u>Acetylene (ml)</u>	<u>Enzyme Solution (ml)</u>
1	0.6	0.0	0.1	0.2	0.5	0.1
2	0.24	0.36	0.1	0.2	0.5	0.1
3	0.15	0.45	0.1	0.2	0.5	0.1
4	0.06	0.54	0.1	0.2	0.5	0.1

After all the reaction vials were set up, 0.1 ml of enzyme solution was injected as per time schedule. The reaction time for all vials was set at 4 minutes. After the designated reaction period, the reaction vial was quenched by the injection of 0.1 ml of 30% trichloroacetic acid. Some 500 micro liters of gaseous phase was taken out to be analyzed by GLC for acetylene/ethylene assay.

f. Determination of Initial Velocities by Rapid Chemical Quench
Method

The diagrammatic apparatus shown in the next page is designed to measure the initial velocities at a much shorter reaction time course (i.e., from 0 to 45 seconds). ATP hydrolysis and acetylene reduction were measured simultaneously for nitrogenase activity at different ATP and ADP concentration levels.

Reagents:

The stock solutions and the reagents used in this experiment were the same as those used in the previous enzyme studies (section e). The enzyme components ratio was also 5.0 to 1.0. (Refer to the tables in section (e) of enzyme studies).

An enzyme solution was placed in syringe "A" after evacuating the syringe three times with argon gas and flushing it with sodium dithionite solution.

ATP and ADP stock solutions, sodium dithionite solution and KHEPES buffer were placed in syringe "B" after acetylene gas was dissolved and equilibrated by vigorous mechanical shaking at room temperature.

One ml of 3% trichloroacetic acid solution was placed in a quenching centrifuge tube.

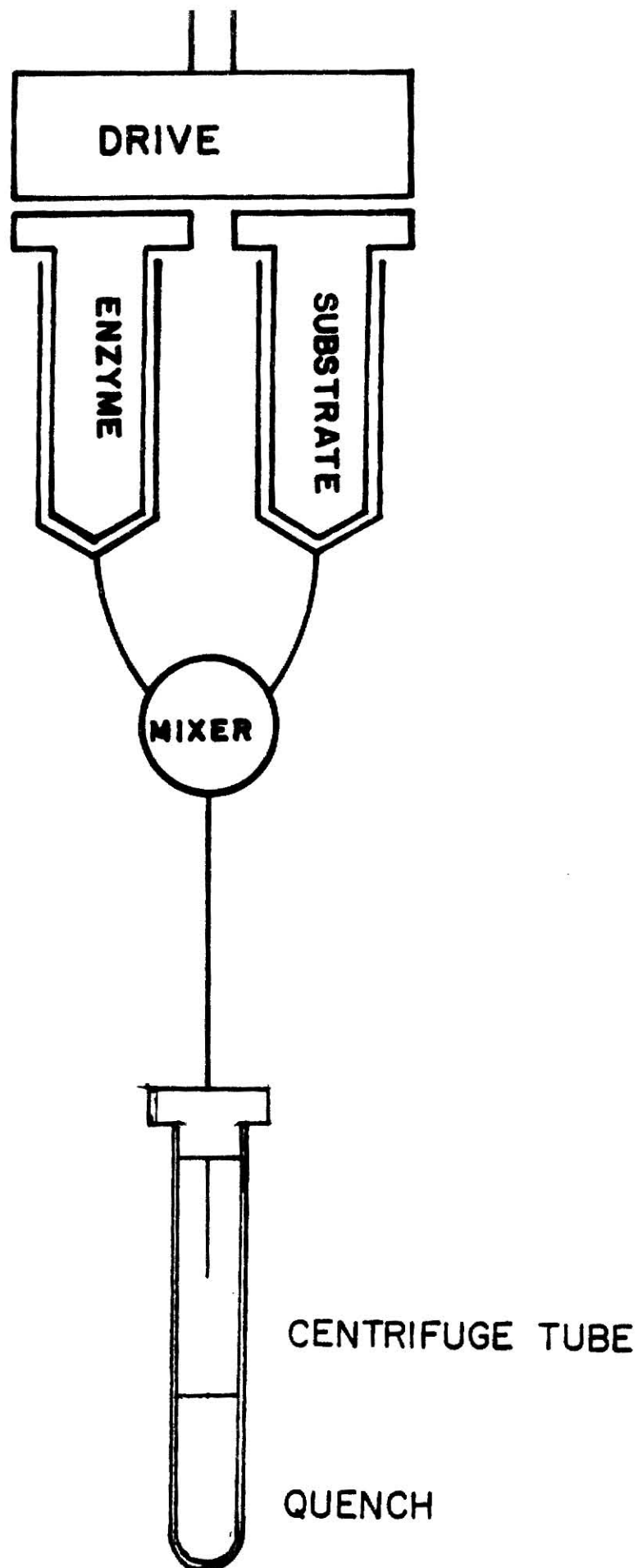
Each shot dispenses 0.11 ml of enzyme solution and 0.11 ml of ATP/ADP solution. After a designated time interval, a second shot displaces the reaction mixture into a quenching centrifuge tube so as to stop the reaction immediately.

The quenching centrifuge tube was centrifuged to make proteins settle

down the wall of the tube. A 0.5 ml of gaseous phase was taken out by a syringe for acetylene assay by GLC; and, 1.0 ml of liquid phase was taken out and placed in 1.1 ml of the mixture of 10 parts of sodium acetate and 1 part of formaldehyde solution plus 0.45 ml of 3% trichloroacetic acid solution. This quenched reaction mixture was used immediately for phosphate assay. Phosphate was assayed by the same method as that described in section (c) of Chemical Methods.

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III. RESULTS

ENZYME STUDIES

a. Mg²⁺ Effect on Nitrogenase Activity

ATP was not hydrolyzed in the absence of Mg²⁺; hence, there was no increase of phosphate. In the presence of Mg²⁺ at 10 mM, 7.5 mM and 5.0 mM, and at fixed ATP concentration of 5.0 mM, the ATP hydrolysis and acetylene reduction were observed as shown in figures a-1 and a-2 respectively. Although ATP was hydrolyzed in the presence of Mg²⁺, a higher Mg²⁺ concentration depressed all these reactions in the later phase of each reaction. However, initial velocities of ATP hydrolysis at different levels of Mg²⁺ appeared identical, and this phenomenon was also common in acetylene reduction and dinitrogen reduction. The graphically obtained initial velocities of each reaction are as follows:

<u>Reaction</u>	<u>Initial Velocities</u>
ATP Hydrolysis	173 n moles/ml · min.
Acetylene Reduction	39.3 n moles/ml · min.
Dinitrogen Reduction	6.7 n moles/ml · min.

This result suggests that Mg²⁺ itself does not have a direct effect on nitrogenase activity. The production of electrons was 43.3 n moles electron pairs per ml · min., and that of dinitrogen reduction was 20 n moles/ml · min. Except hydrogen reduction, the percentage of electron utilization by acetylene reduction was 90.8% and that by dinitrogen reduction was only 46.2% at an energy charge near 1.0, which was a much higher level than the 0.85 energy charge of a usual cell.

Legend to Figures (a-1)

and (a-2)

Mg²⁺ Effect on ATP Hydrolysis

Figure (a-1) shows the time course of ATP hydrolysis at a fixed ATP concentration of 5 mM and magnesium concentrations of 0, 5, 7.5 and 10 mM in the absence of ADP.

Mg²⁺ Effect on Acetylene Reduction

Figure (a-2) shows the time course of acetylene reduction at a fixed ATP concentration of 5 mM and at different Mg²⁺ concentration levels - 5.0, 7.5 and 10 mM.

ATP Hydrolysis & H₂ Evolution

○	5 mM Mg ²⁺ , 5 mM ATP
□	7.5 mM Mg ²⁺ , 5 mM ATP
Δ	10 mM Mg ²⁺ , 5 mM ATP

FIG. a-1

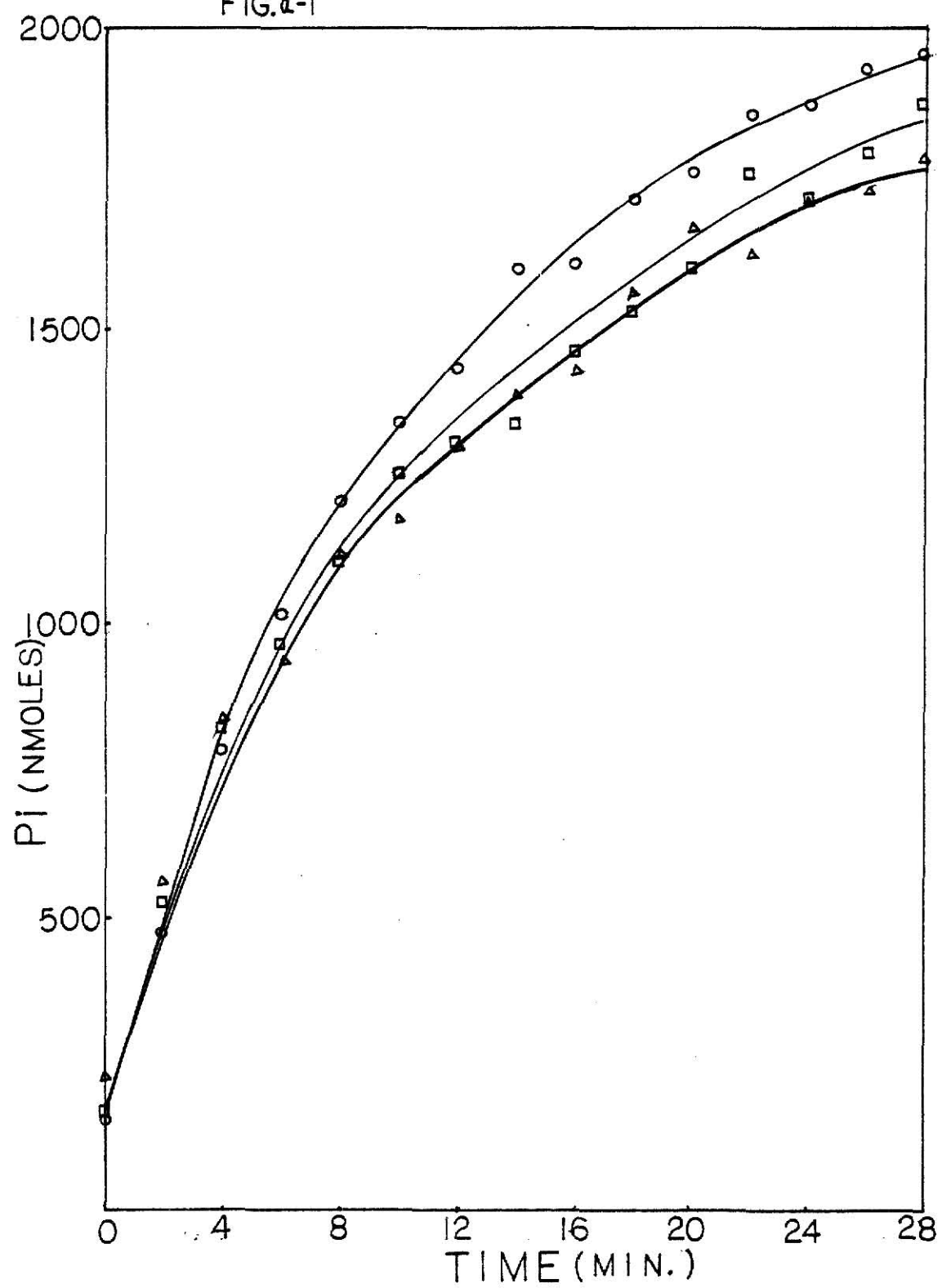
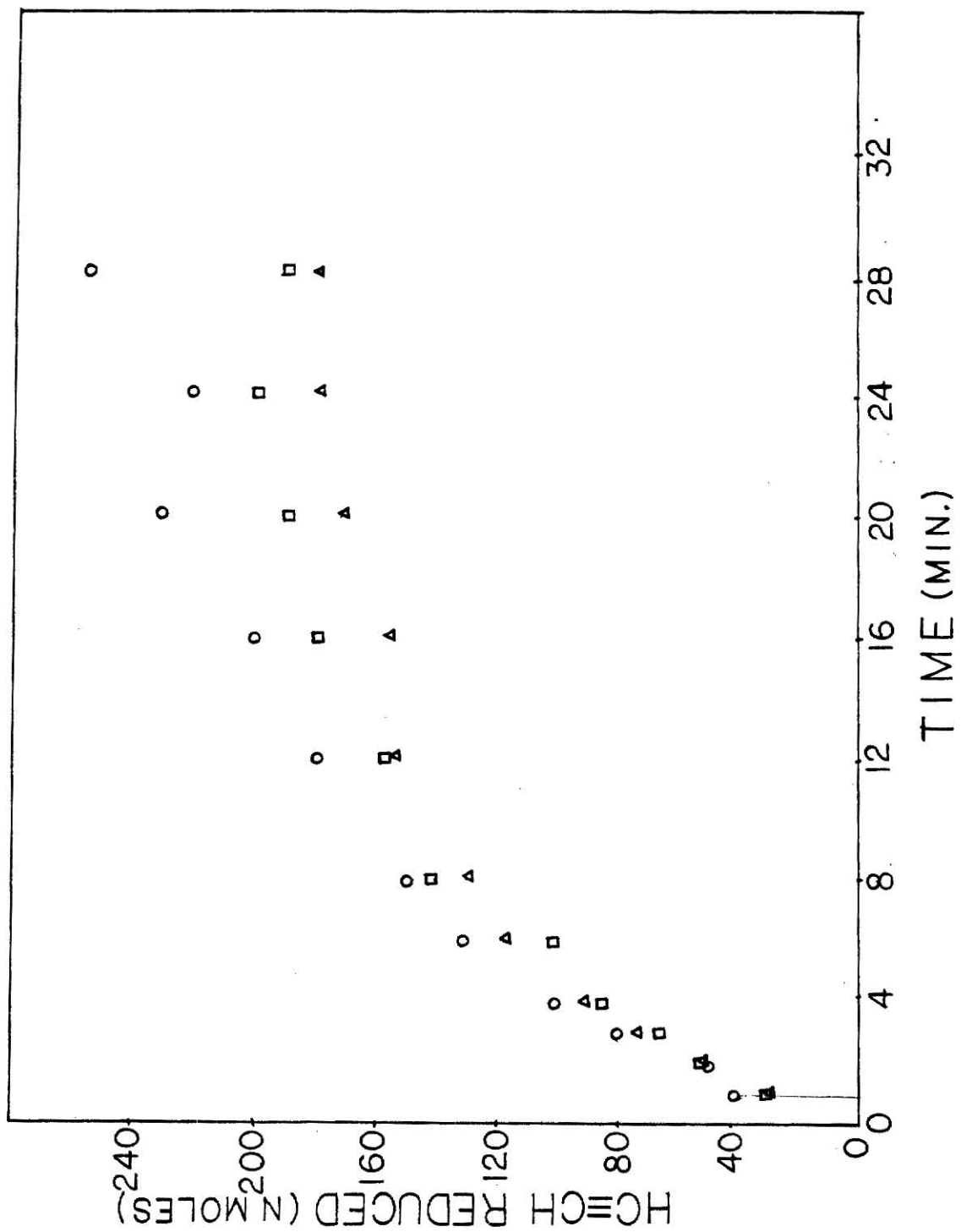


FIG. a-2



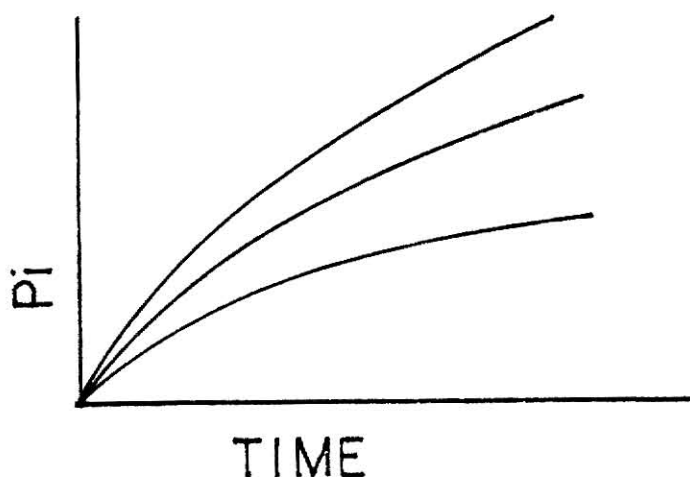
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b. ADP Effect on Nitrogenase Activity

As can be seen in figures (b-1) through (b-4), higher levels of ADP inhibit ATP hydrolysis, acetylene reduction and dinitrogen reduction very strongly. The results of ADP effect can be the sum of two effects: (1) the change of energy charge, or (2) ADP as an inhibitor. Therefore, apparent time course curves were the integrated effects of ADP which reduces initial energy and inhibits nitrogenase activity. The examined ADP concentrations were 0.0 mM, 0.2 mM, 0.4 mM and 0.6 mM. The energy charge was changed from 1.0 to 0.89. The time course curves seem to be linear during the examined time period and appeared as zero-order velocity curves.

The result also suggests that the other product of ATP hydrolysis, phosphate, may not have an effect on nitrogenase activity, since there was no significant change in velocities in the time course curves during the examined time period. If phosphate was an inhibitor enough to change the velocity, the earlier phase of higher energy charge curve will be effected more in as much as the accumulation of phosphate would be greater than that of lower energy charge case and could effect nitrogenase activity in the earlier phase of the time course curve. The expected time course curves might be as that in the graph shown below.



LEGEND to Figures (b-1), (b-2), (b-3) & (b-4)

ADP Effect on ATP Hydrolysis, H₂ Evolution, Acetylene Reduction and Dinitrogen Reduction

The following figures show the effect of ADP on ATP hydrolysis (fig. b-1), H₂ evolution (fig. b-2), acetylene reduction (fig. b-3) and dinitrogen reduction (fig. b-4) at fixed Mg²⁺ and ATP concentrations (10 mM and 5 mM respectively). The ratios of ATP/(ATP + ADP) were 1.0, 0.96, 0.93 and 0.89.

Note: ATP hydrolysis, hydrogen evolution and dinitrogen reduction were assayed simultaneously. The acetylene reduction time course curve was obtained from another experiment under the same experimental conditions except argon ambient instead of dinitrogen ambient.

The following table shows the examined ADP, ATP and Mg²⁺ concentrations.

<u>ADP</u> <u>Concentration</u>	<u>ATP</u> <u>Concentration</u>	<u>Mg²⁺</u> <u>Concentration</u>	<u>Energy</u> <u>Charge</u>
● ... 0.0 mM	5 mM	10 mM	1.0
□ ... 0.2 mM	5 mM	10 mM	0.96
◇ / △ ... 0.4 mM	5 mM	10 mM	0.93
○ ... 0.6 mM	5 mM	10 mM	0.89

FIG.b-1

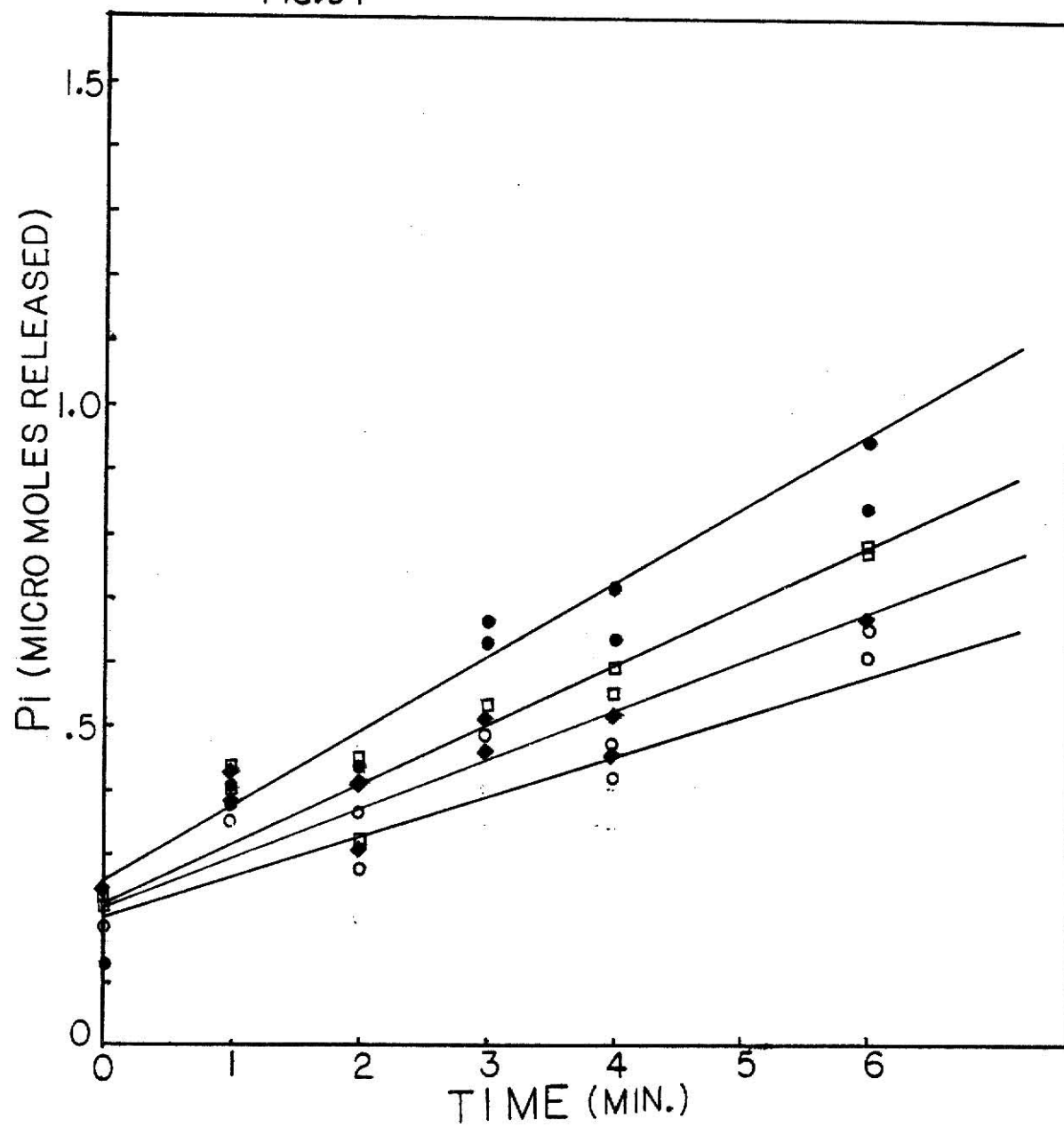


FIG.b-2

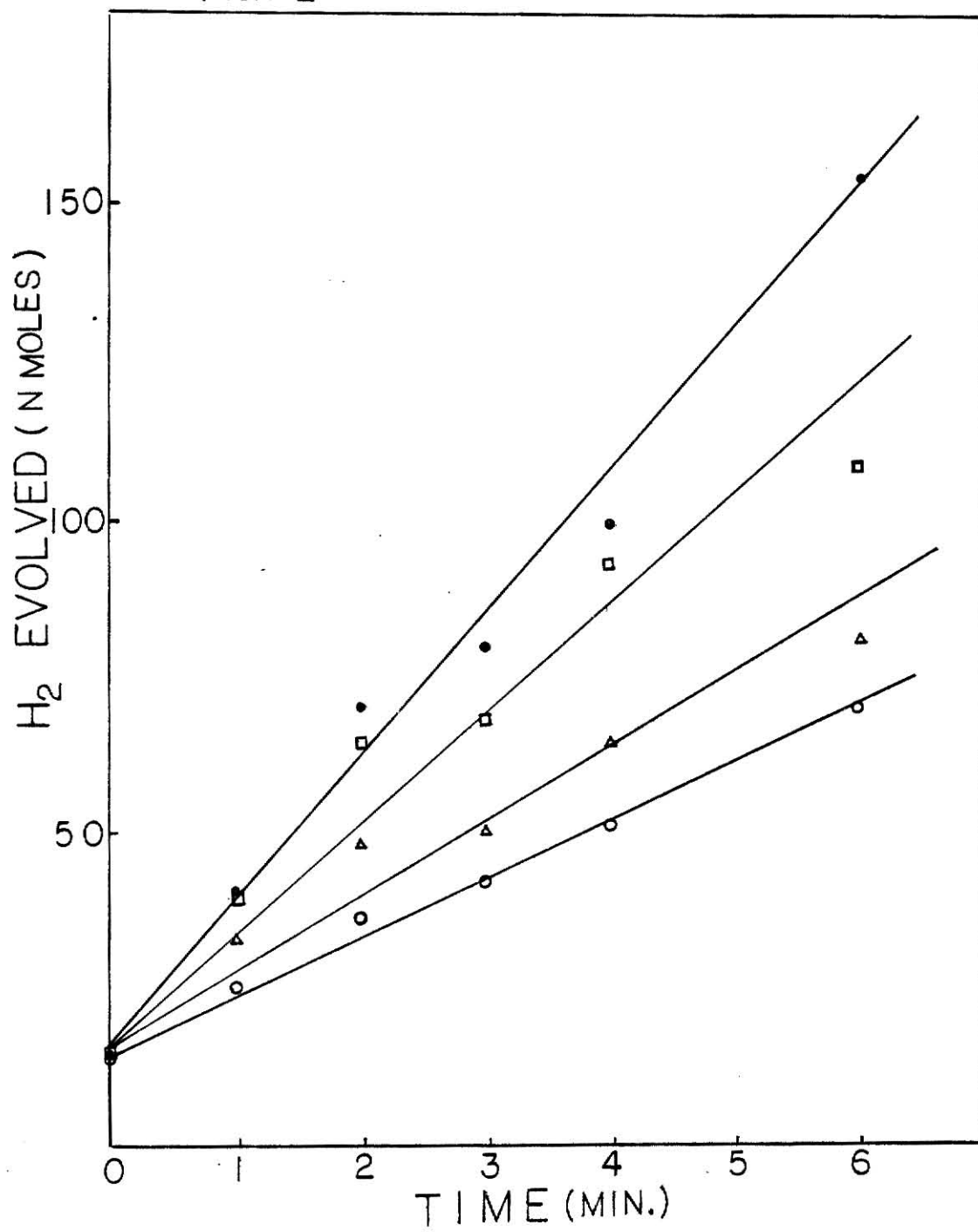


FIG.b-3

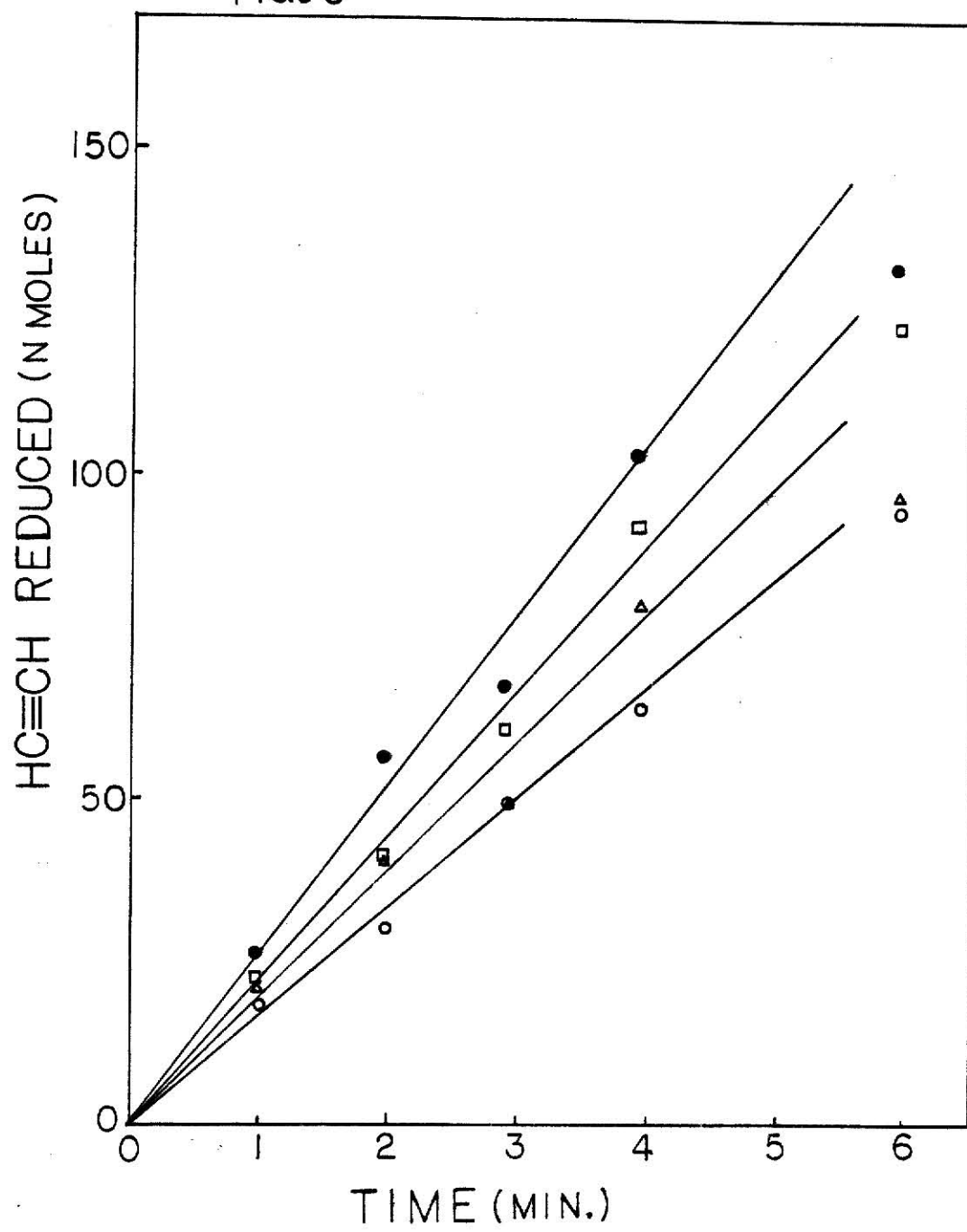
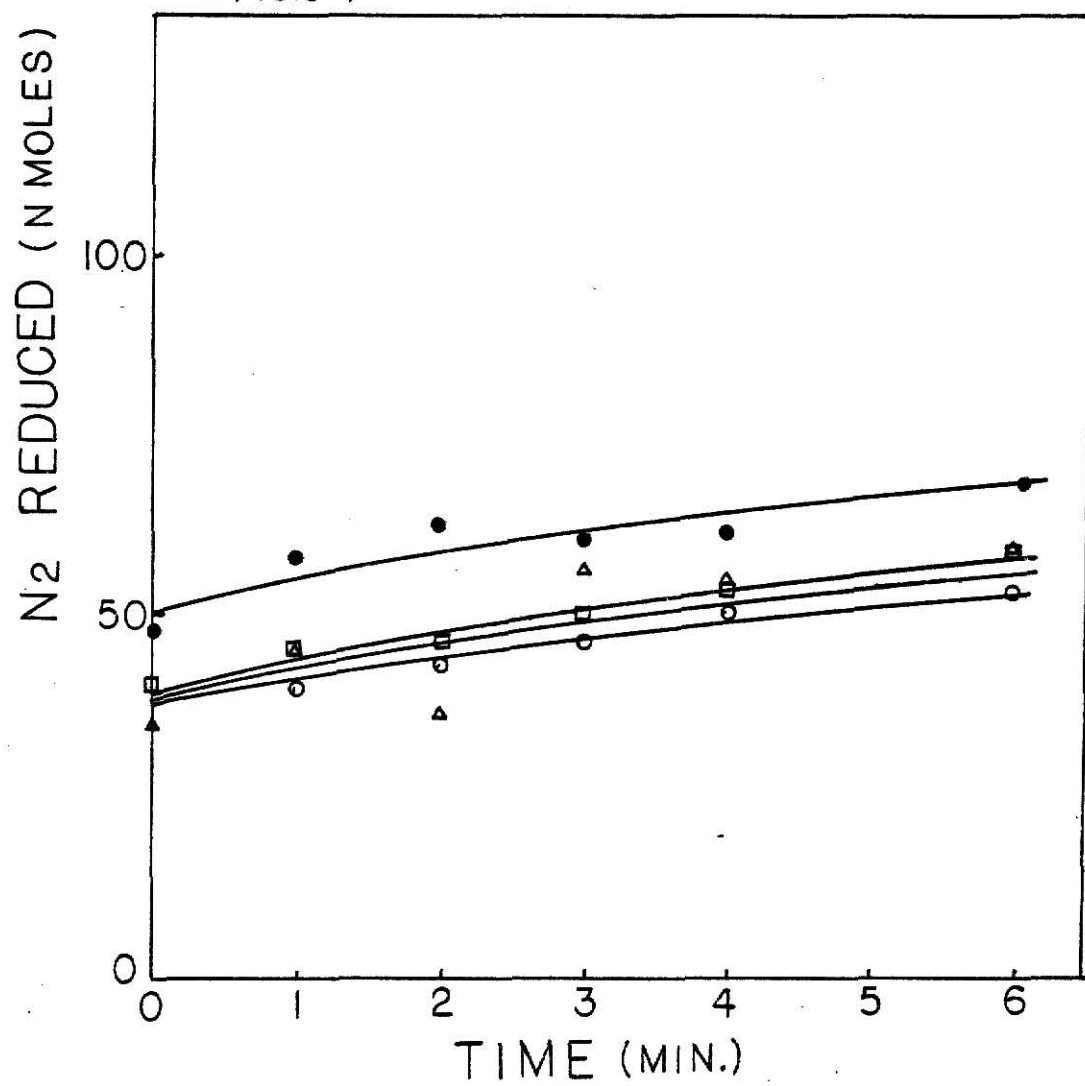


FIG.b-4



c. ATP Effect on Nitrogenase Activity

Figures (c-1) through (c-4) show the effect of ATP concentrations on nitrogenase activity. Higher levels of ATP give greater initial velocities and seem to keep the linearity of initial velocity for a longer period of time, whereas lower concentration of ATP not only gives smaller initial velocity but also loses its initial velocity at the earlier phase of the time course curve. This result suggests that the product of ATP hydrolysis, ADP, inhibits ATP hydrolysis and the successive reduction reactions. The result might be consistent to that of enzyme studies (b), where there was already a designated amount of ADP in the reaction mixture and the initial velocities were changed. On the other hand, in enzyme studies (c), the initial velocities were only a function of original ATP concentration and resulted in corresponding degree of initial velocities. However, the reaction proceeded, and the production of ADP could not be ignored since even a slight accumulation of ADP could change the energy charge drastically. In comparison with enzyme studies (b) where a slight change in ADP per original ADP concentration could be ignored, a slight increase of ADP in enzyme studies (c), where originally there was no ADP in the reaction mixture, effects nitrogenase activity. This is especially so in the case where the ATP concentration was lower. The ratio ADP/ATP could be changed very drastically by a slight increase in ADP production during ATP hydrolysis. This may be the reason why the time course curve at a lower ATP concentration lost the initial velocity at an earlier phase of the time course.

This result also suggests that ADP may be a very potent inhibitor and may possess very low K_i value compared to the K_m value of ATP hydrolysis.

LEGEND to Figures (c-1) through (c-4)

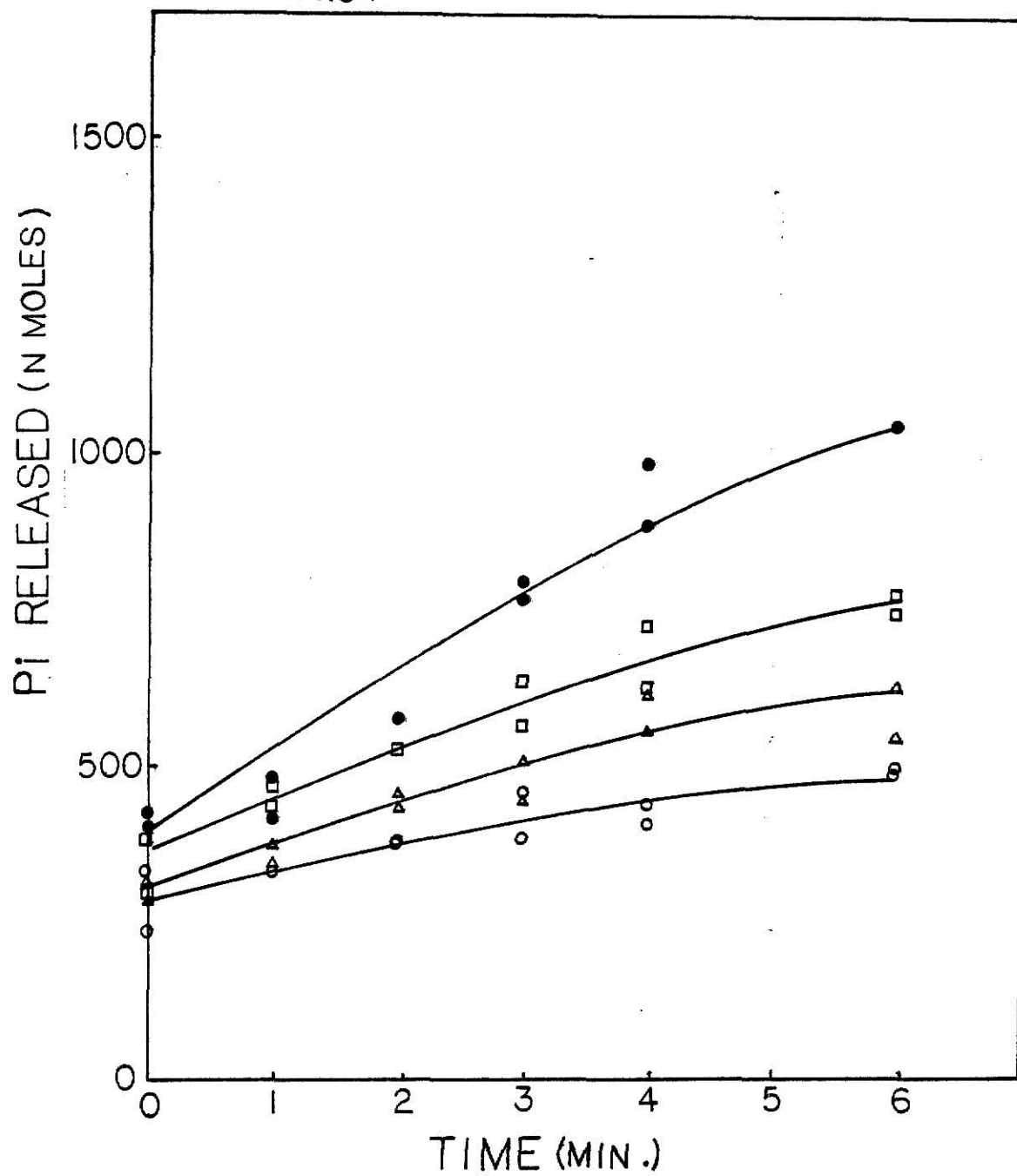
ATP Effect on ATP Hydrolysis, H₂ Evolution, Acetylene Reduction and
Dinitrogen Reduction

The following figures (c-1, c-2, c-3, c-4) show the time course study of the effects of ATP on ATP hydrolysis, H₂ evolution and acetylene reduction at a fixed level of Mg²⁺ (10 mM) and in the absence of ADP.

Note: ATP hydrolysis, H₂ evolution and acetylene reduction were assayed simultaneously. The nitrogen reduction time course was obtained from another experiment under the same experimental conditions except nitrogen ambient.

	<u>ATP (mM)</u>
●	5
□	2
Δ	1.25
○	0.5

FIG. C-1



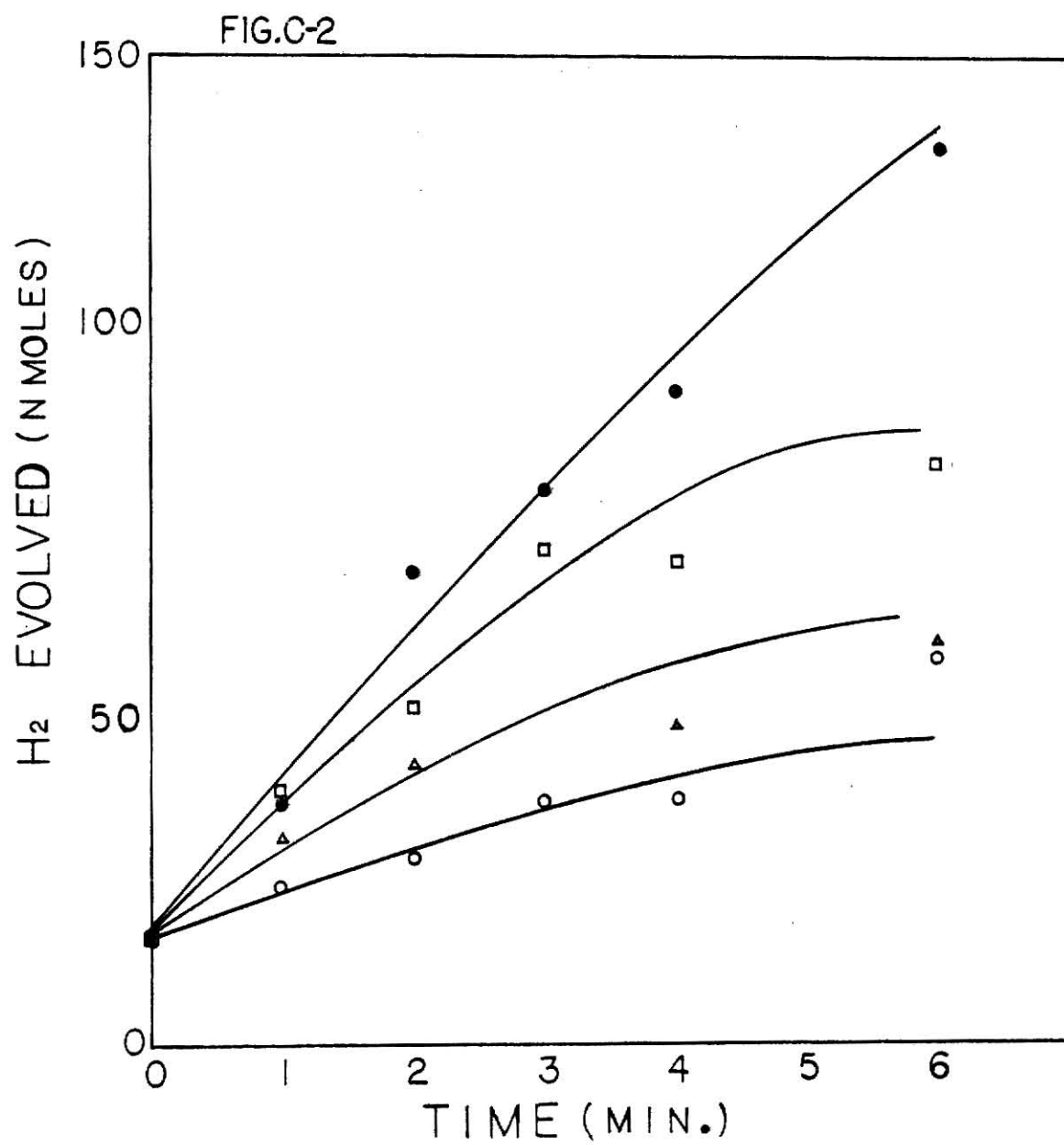


FIG.C-3

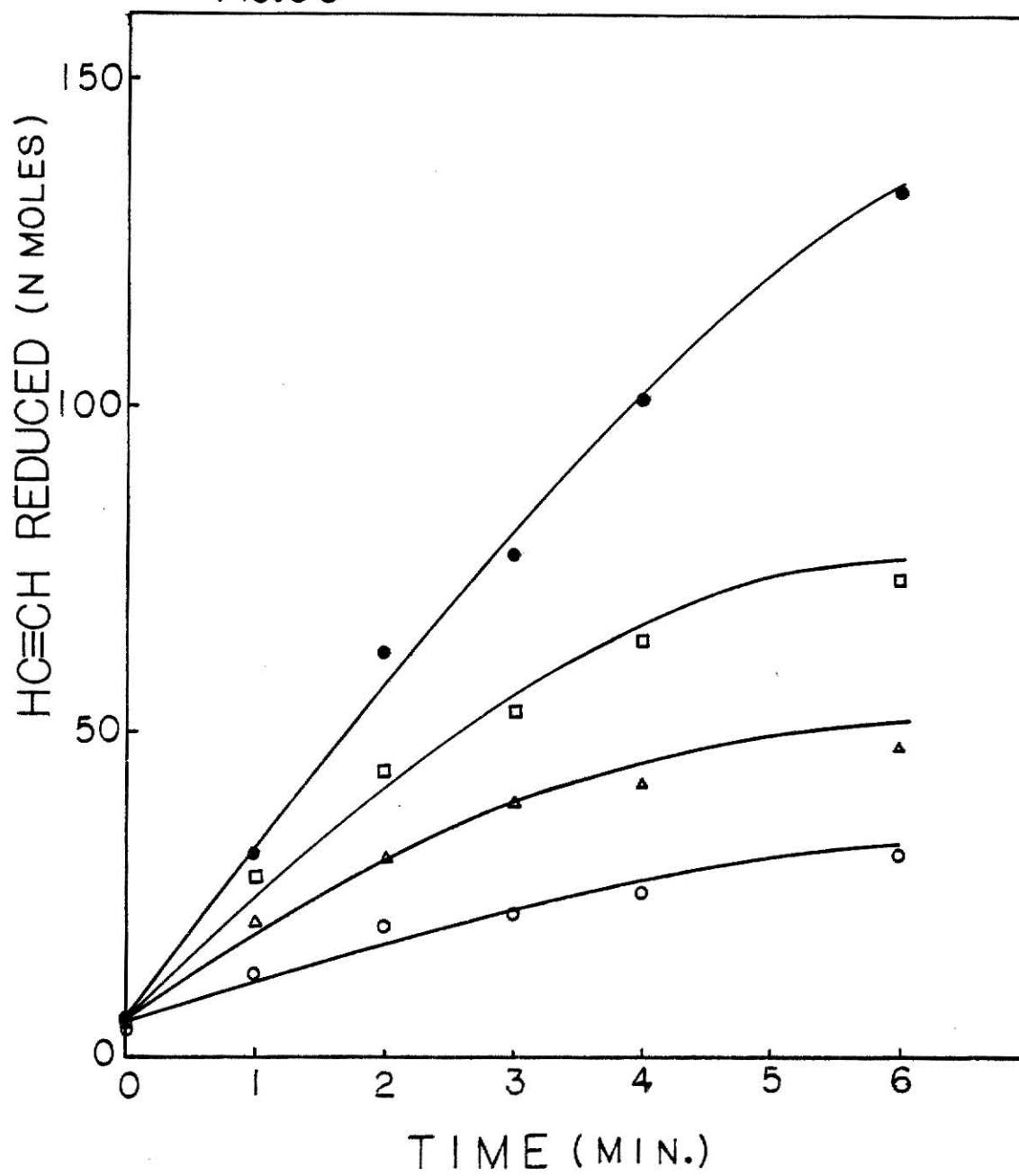
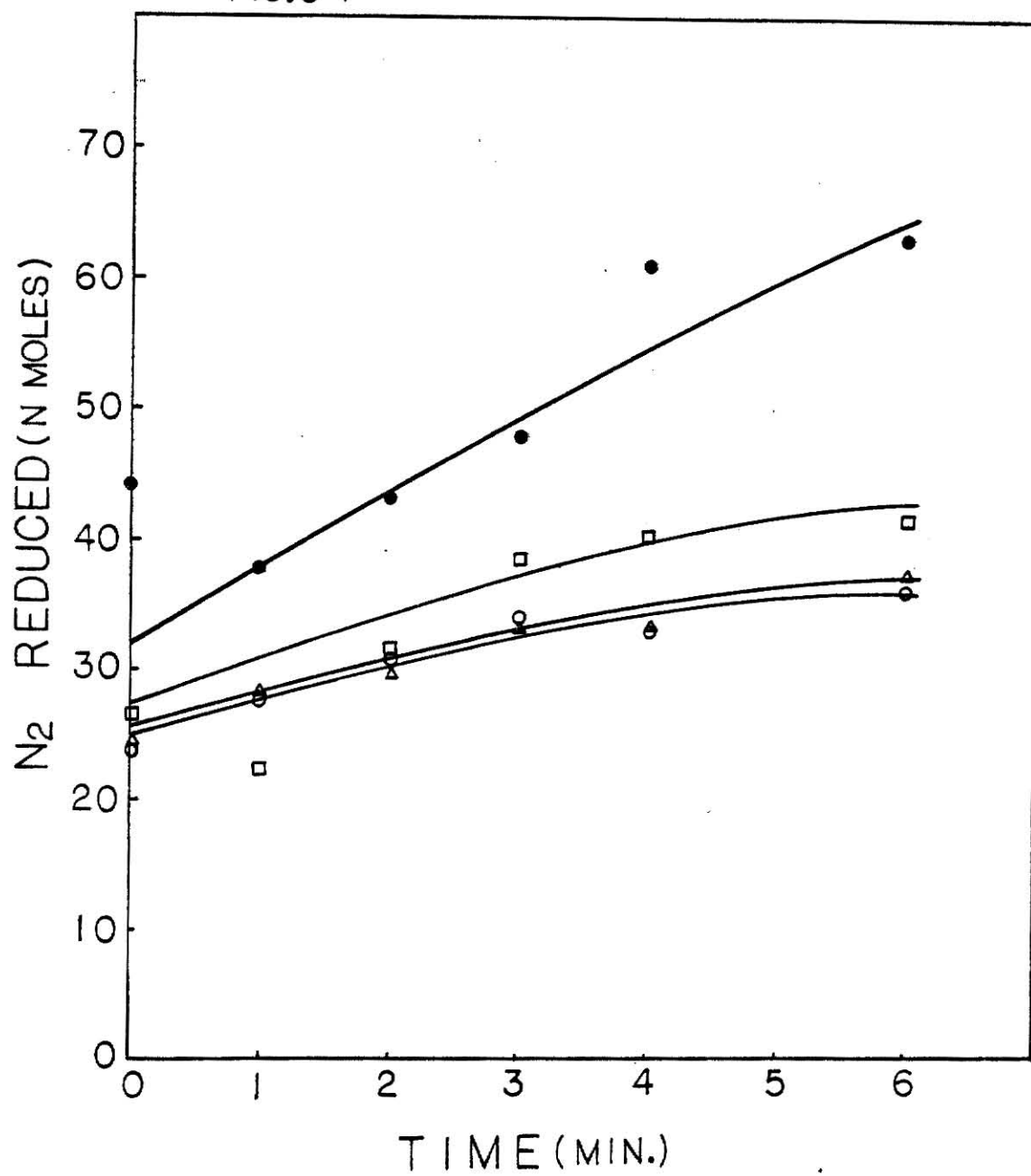


FIG.C-4



d. ADP/ATP Ratio Effect on Nitrogenase Activity

The curves of Lineweaver-Burk plots of ATP hydrolysis, acetylene reduction and hydrogen evolution appeared to be linear as shown in the corresponding figures (d-1) through (d-3). The calculated values of the apparent K_m for ATP of each reaction are as follows:

ATP Hydrolysis: $K_m \text{ app.} = 1.67 \text{ mM}$

H_2 evolution : $K_m \text{ app.} = 1.00 \text{ mM}$

Acetylene reduction: $K_m \text{ app.} = 3.6 \text{ mM.}$

The Dixon plot curves of ATP hydrolysis, hydrogen evolution and acetylene reduction indicate a linear mixed type inhibition pattern. The graphically obtained K_i values for each reaction are as follows:

ATP Hydrolysis : $K_i = 0.59 \text{ mM}$

Hydrogen evolution : $K_i = 0.85 \text{ mM}$

Acetylene reduction: $K_i = 0.48 \text{ mM.}$

Note: K_i values were obtained from Dixon plots.

All K_i 's are smaller than the K_m values, which suggests that ADP is a strong product inhibitor for these reactions.

Under enzyme studies (d), the total adenine nucleotide concentration was fixed at 5 mM. Therefore, energy charge were varied from 0.89 to 1.0 as in enzyme studies (b).

The abscissa denotes the rate of electron utilization in each reaction except that one of ATP hydrolysis where it denotes the rate of electron production available for successive hydrogen evolution and acetylene reduction.

LEGEND to Figures (d-1), (d-2), (d-3) & (d-4)

ADP/ATP Ratio Effect on ATP Hydrolysis and H₂ Evolution

Figures (d-1) and (d-3) show the Lineweaver-Burk plot of the initial velocities of ATP hydrolysis vs $1/\text{ATP}$ (fig. d-1) and the initial velocities of H₂ evolution vs $1/\text{ATP}$ (fig. d-3) at different levels of ADP concentration.

The curves appeared to be linear and it can be observed that ADP influences the intercepts on the abscissa as well as the slopes.

The calculated values of apparent K_m , K_i , K_{is} and K_{ii} are as follows:

	<u>ATP Hydrolysis (mM)</u>	<u>H₂ Evolution (mM)</u>
K_m app.	1.67	1.00
K_i app.	0.59	0.85
K_{is}	0.40	0.62
K_{ii}	0.67	0.60

Note: K_{ii} and K_{is} values were obtained by replotting the intercepts and slopes from the Lineweaver-Burk plots vs ADP concentrations.

Figures (d-2) and (d-4) show the Dixon plot of the initial velocities of ATP hydrolysis vs $1/\text{ATP}$ (fig d-2) and the initial velocities of hydrogen evolution vs $1/\text{ATP}$ (fig. d-4) at different levels of ATP concentrations.

<u>L-B plot</u>	<u>Dixon plot</u>
● 0.0 mM ADP	5.00 mM ATP
□ 0.2 mM	2.00 mM
△ 0.4 mM	1.25 mM
○ 0.6 mM	0.50 mM

FIG.d-1

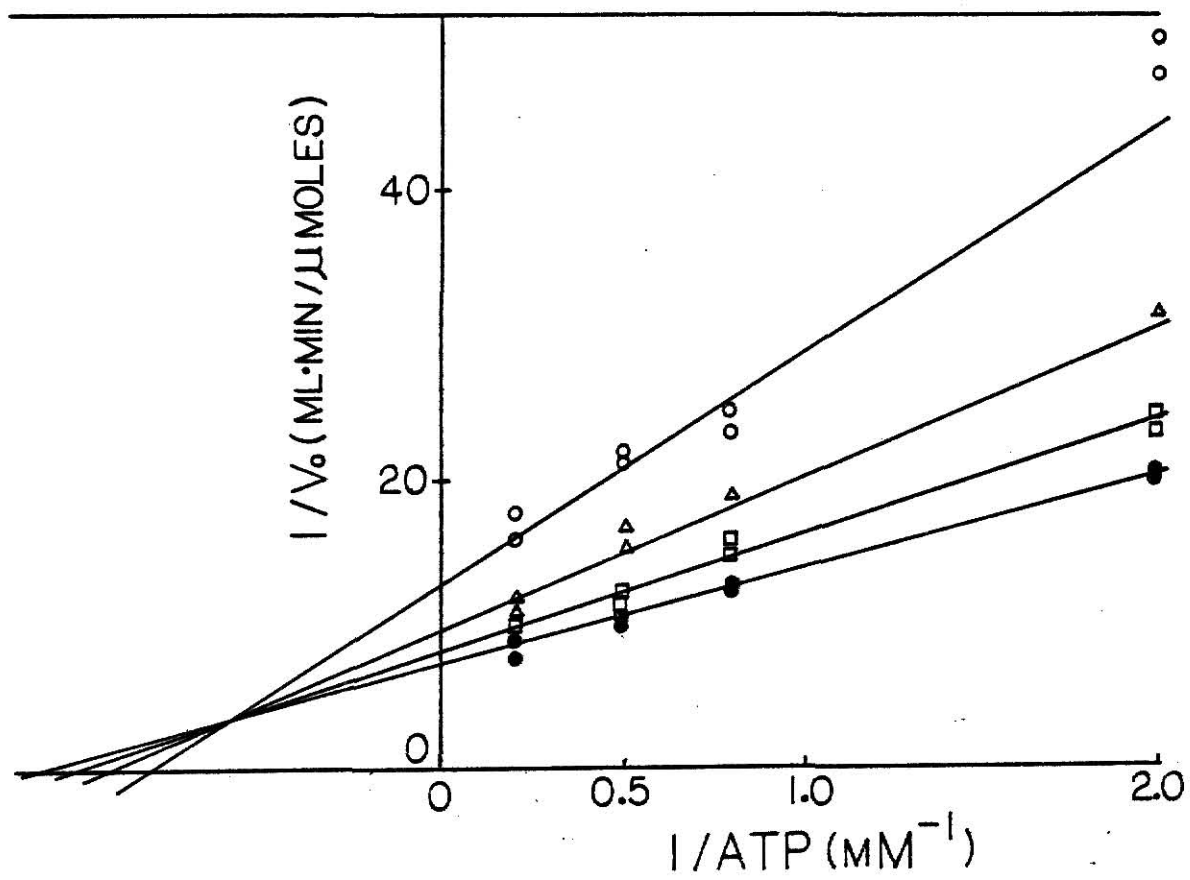


FIG. 4-2

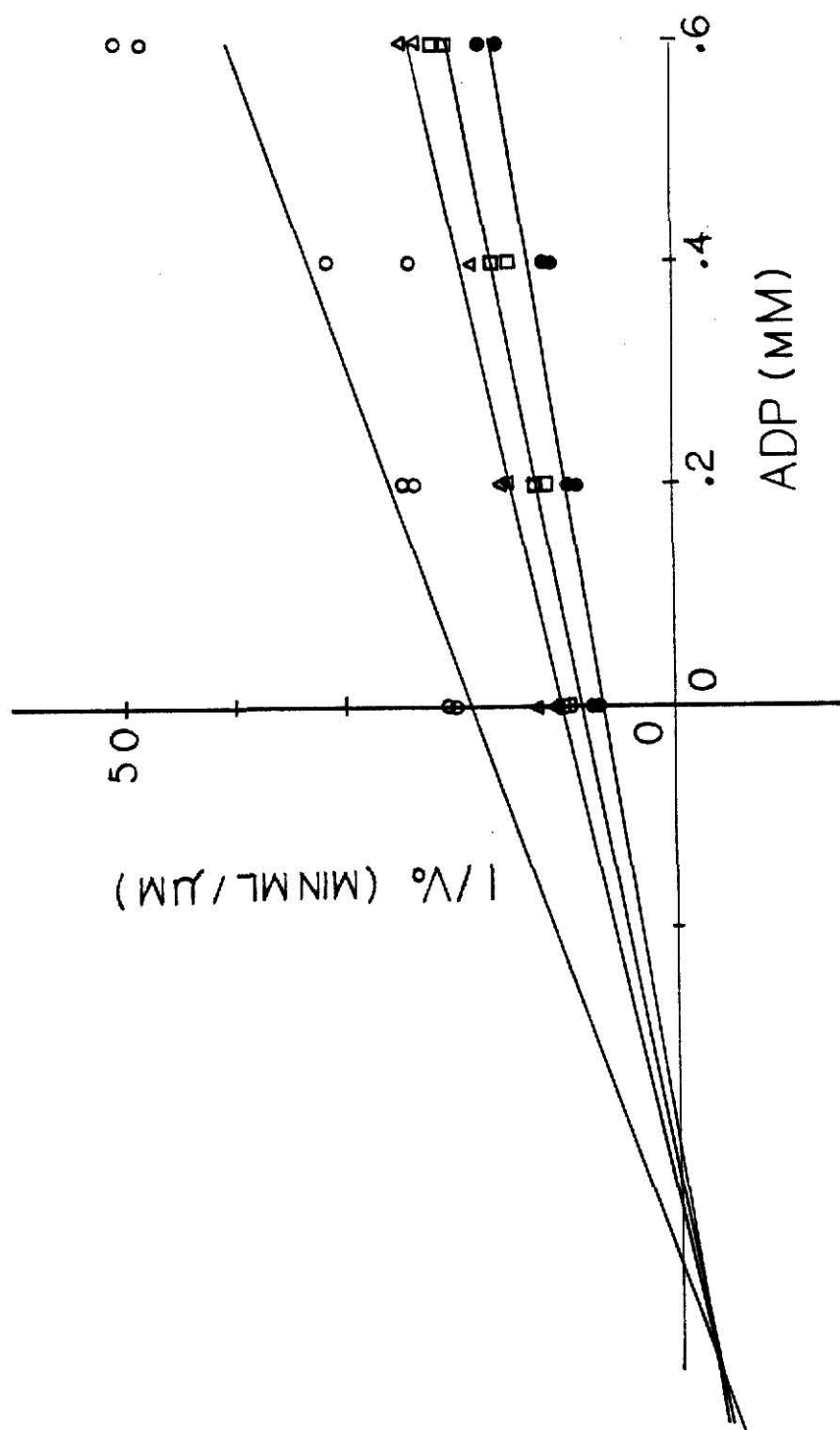


FIG.d-3

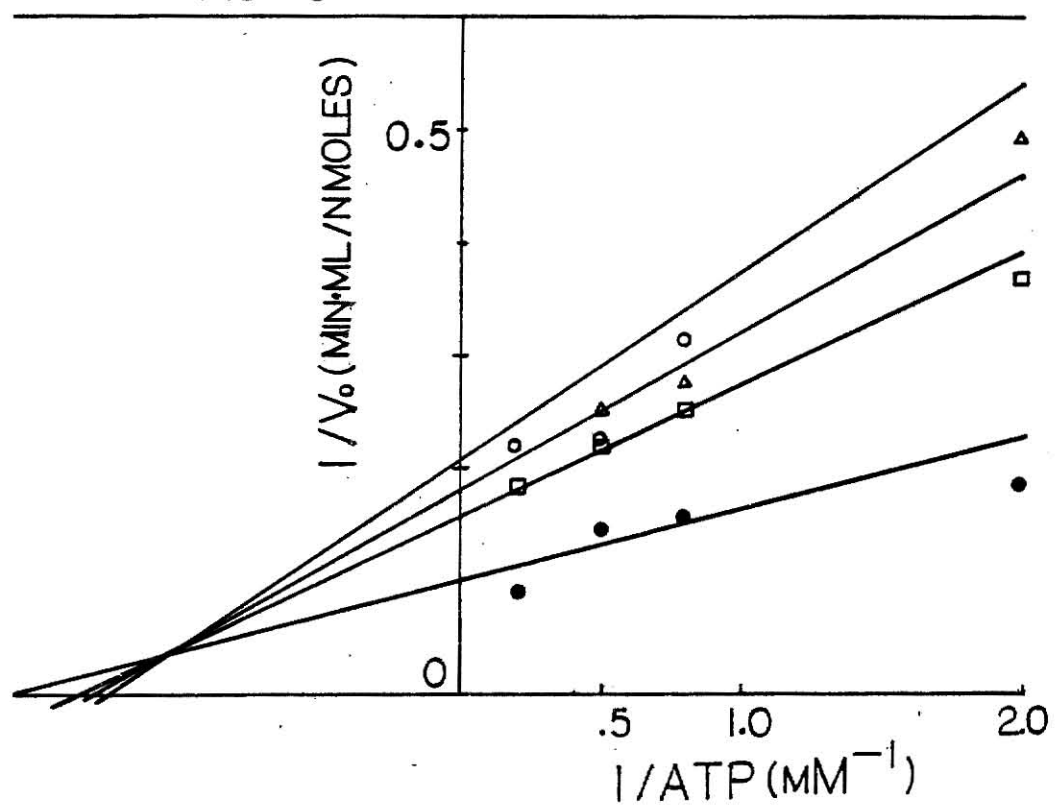
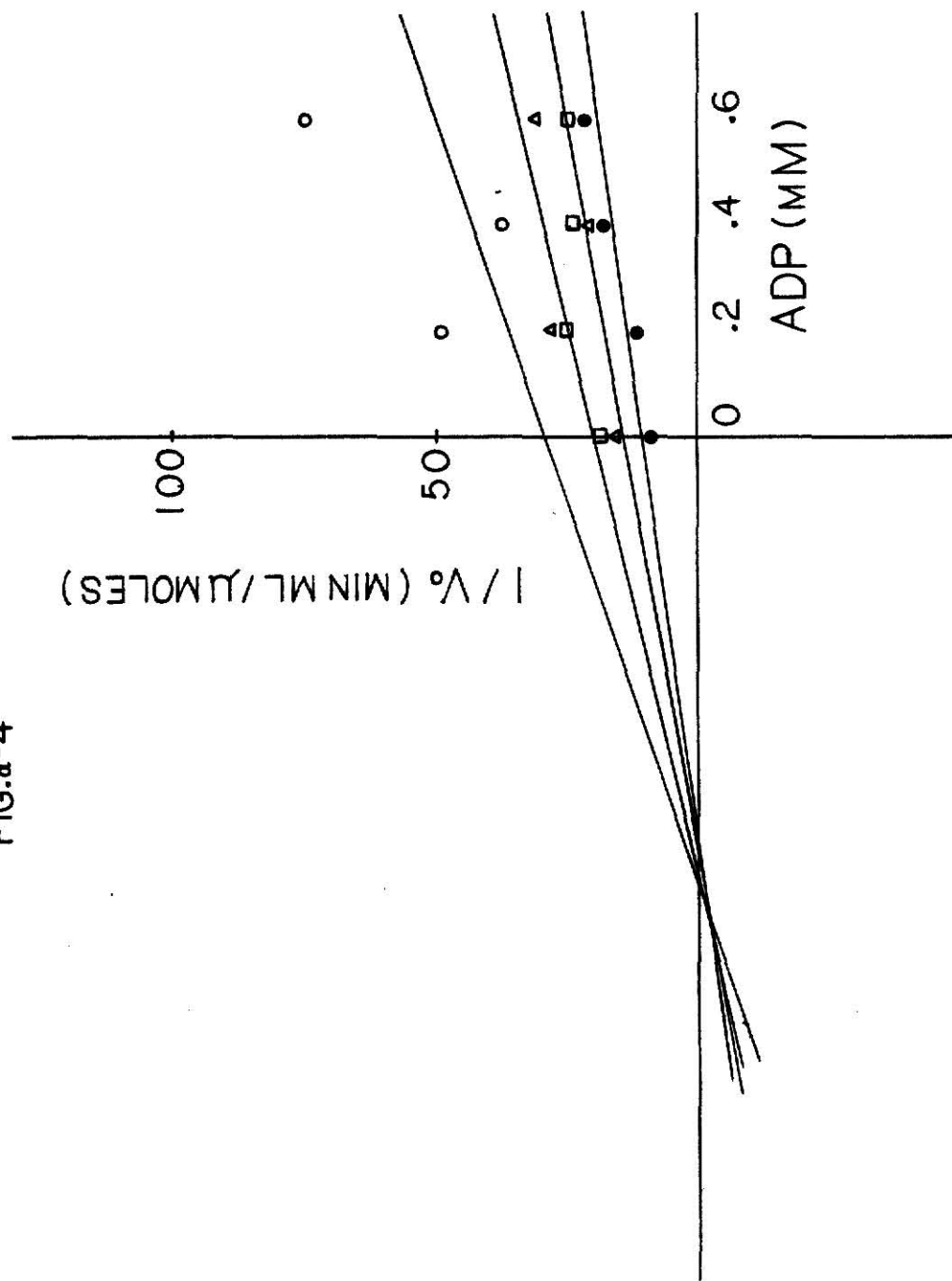


FIG.d-4



LEGEND to Figures (d-5) & (d-6)

ADP/ATP Ratio Effect on Acetylene Reduction

Figure (d-5) shows the Lineweaver-Burk plot of the initial velocities of acetylene reduction vs $1/\text{ATP}$ at different levels of ADP concentration. The curves seem to be linear and different levels of ADP influence both the slopes and the intercept values.

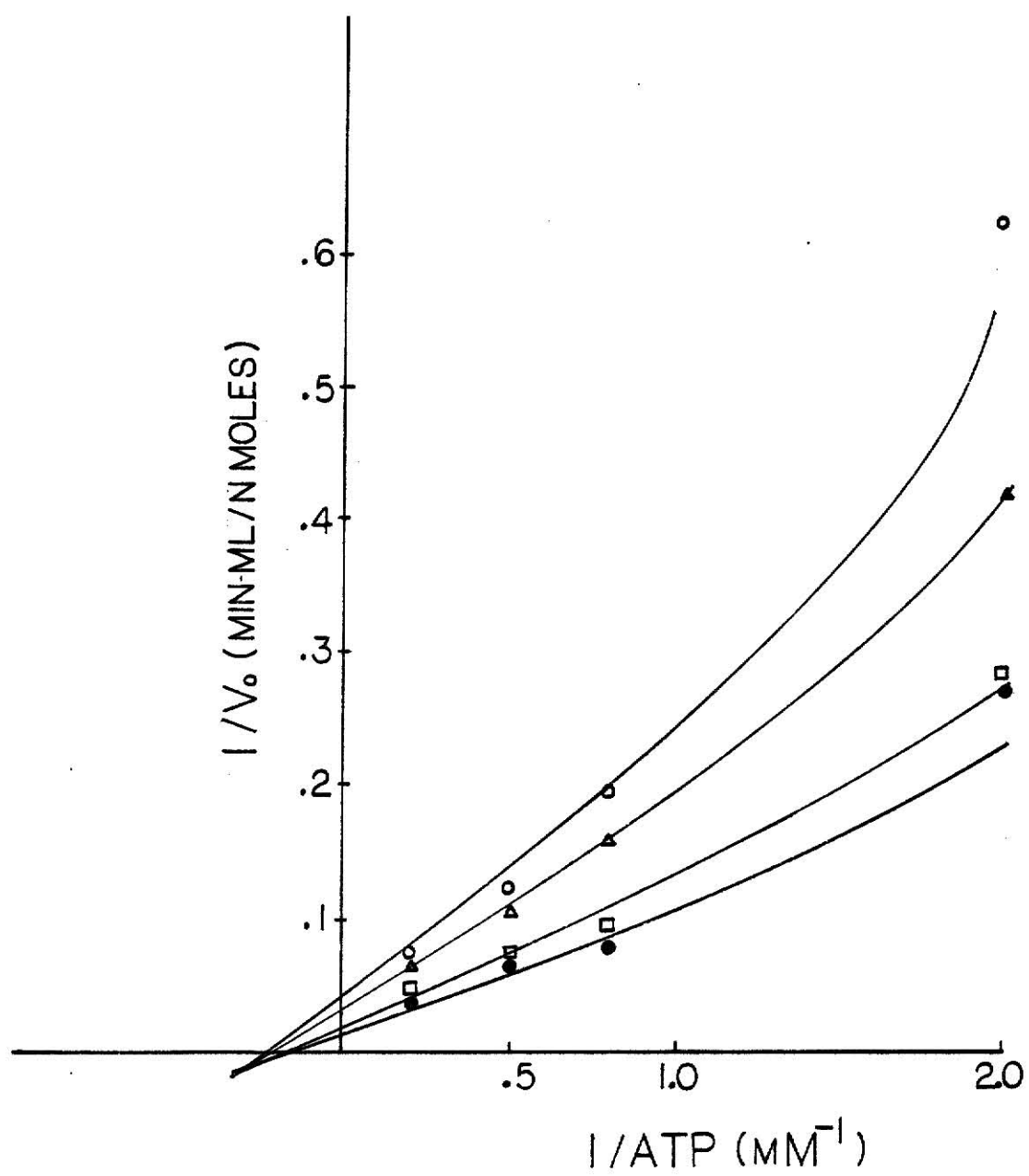
Figure (d-6) shows the Dixon plot of acetylene reduction. The calculated values of apparent K_m , K_i , K_{is} and K_{ii} are as follows:

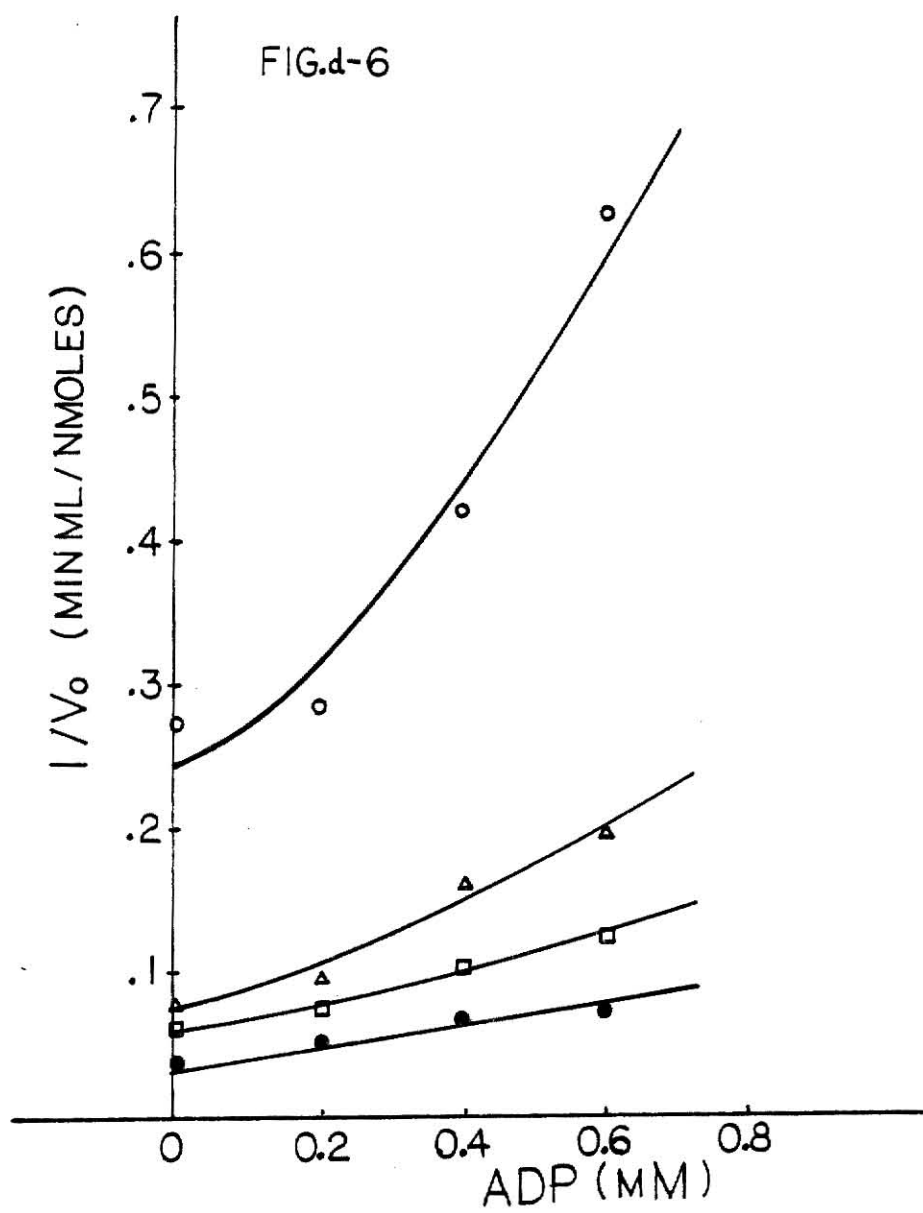
	<u>Acetylene reduction</u>
K_m app.	3.60 mM
K_i app.	0.48 mM
K_{is}	0.58 mM
K_{ii}	0.70 mM

Note: K_{is} and K_{ii} values were obtained from the replots of slopes and intercepts of Lineweaver-Burk plot vs. ADP concentrations.

○	0.6	mM ADP
△	0.4	"
□	0.2	"
●	0.0	"

FIG.d-5





e. Ratio Effect of Component 2 to Component 1 on Nitrogenase Activity

In enzyme studies (e), a higher component ratio of 5.0 to 1.0 (component 2/component 1) was used, and the graphically obtained K_m and K_i values for ATP hydrolysis and hydrogen evolution were not changed compared to those obtained in enzyme studies (c) where the component ratio was 2.5 to 1.0.

The K_i and K_m values for acetylene reduction were drastically changed. The inhibition pattern appeared as a mixed type of inhibition except ATP hydrolysis. These observations show that Fe protein (component 2) and MoFe protein (component 1) might exist at equilibrium. Thus, at a higher ratio of component 2 to component 1, it gave a higher rate of ATP hydrolysis, since Fe protein might behave as if it were a substrate carrying electron to MoFe protein.

The calculated value of apparent V_{max} of ATP hydrolysis at the component ratio of 2.5/1.0 and 5.0/1.0 were as follows:

	<u>Ratio</u>	
	<u>2.5/1.0</u>	<u>5.0/1.0</u>
ATP Hydrolysis	184 n moles/min·ml	280 n moles/min·ml

where n moles are of phosphate produced during ATP hydrolysis.

1
LEGEND to Figure (e-1)

Ratio Effect of Component 2 to Component 1 on ATP Hydrolysis

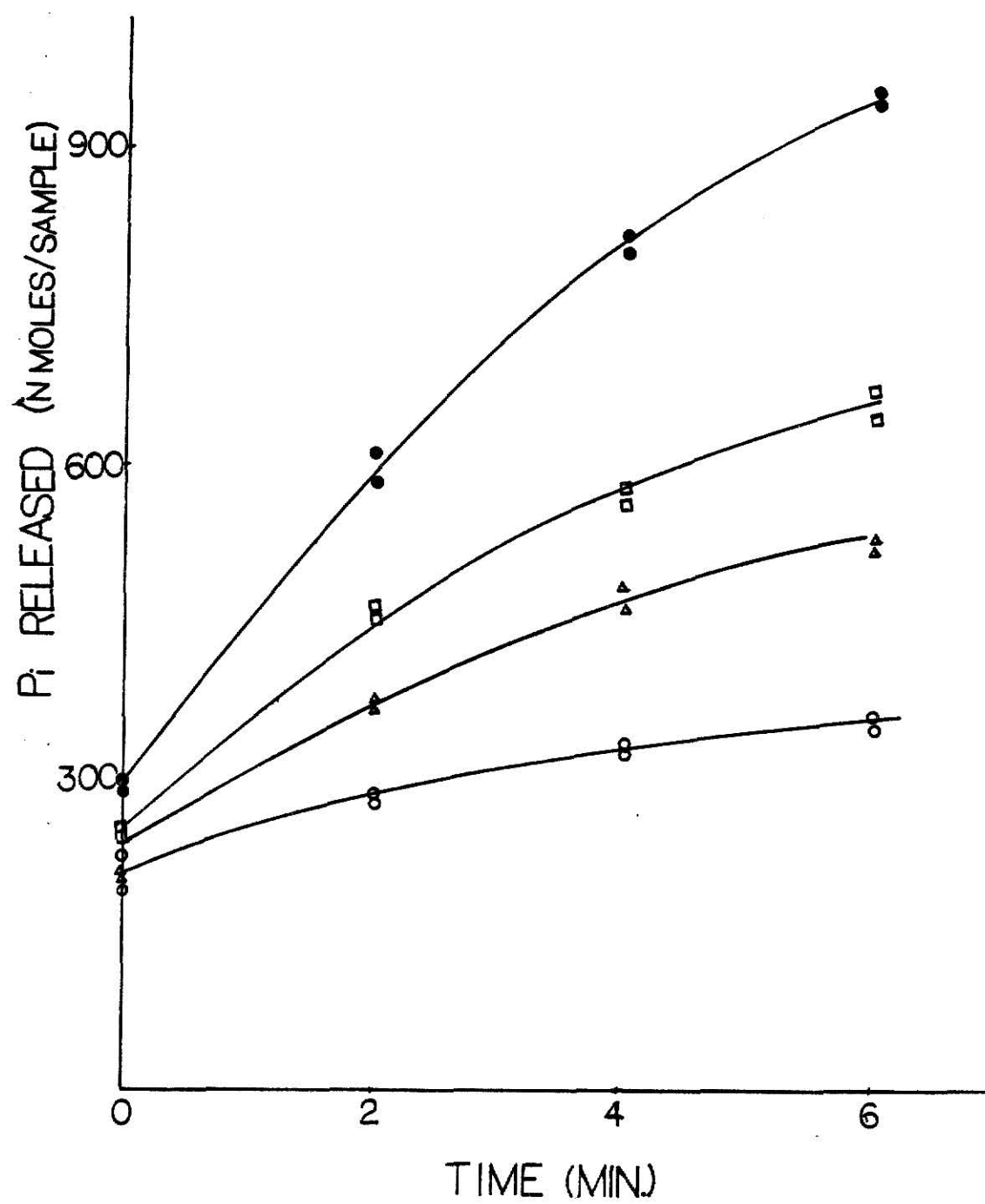
Figure (e-1) shows one of the time courses of ATP hydrolysis at a fixed ATP level and at different ADP levels of 0.0 mM to 0.6 mM.

From this time course analysis, the initial velocities (in n moles/min · ml.) were obtained as follows:

<u>ATP level (mM)</u>	<u>ADP levels (mM)</u>			
	<u>0.0</u>	<u>0.2</u>	<u>0.4</u>	<u>0.6</u>
0.5	84.0	59.0	54.0	28.5
1.25	98.0	66.0	54.0	39.5
2.0	152.0	102.0	85.5	43.5
5.0	186.0	156.0	93.5	59.0

- 0.0 mM ADP
- 0.2 "
- △ 0.4 "
- 0.6 "

FIG. e-1



LEGEND to Figures (e-2) and (e-3)

Ratio Effect of Component 2 to Component 1 on ATP Hydrolysis

Figures (e-2) and (e-3) show the Lineweaver-Burk plot and Dixon plot of ATP hydrolysis respectively. Mg^{2+} concentration was fixed at 10 mM.

The graphically obtained K_m and K_i values of ATP Hydrolysis from Lineweaver-Burk and Dixon plots were:

$$K_m = 1.70 \text{ mM}$$

$$K_i = 0.48 \text{ mM}$$

	<u>L-B plot (ADP mM)</u>	<u>Dixon plot (ATP mM)</u>
●	0.0	5.0
□	0.2	2.0
Δ	0.4	1.25
○	0.6	0.5

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FIG.e-2

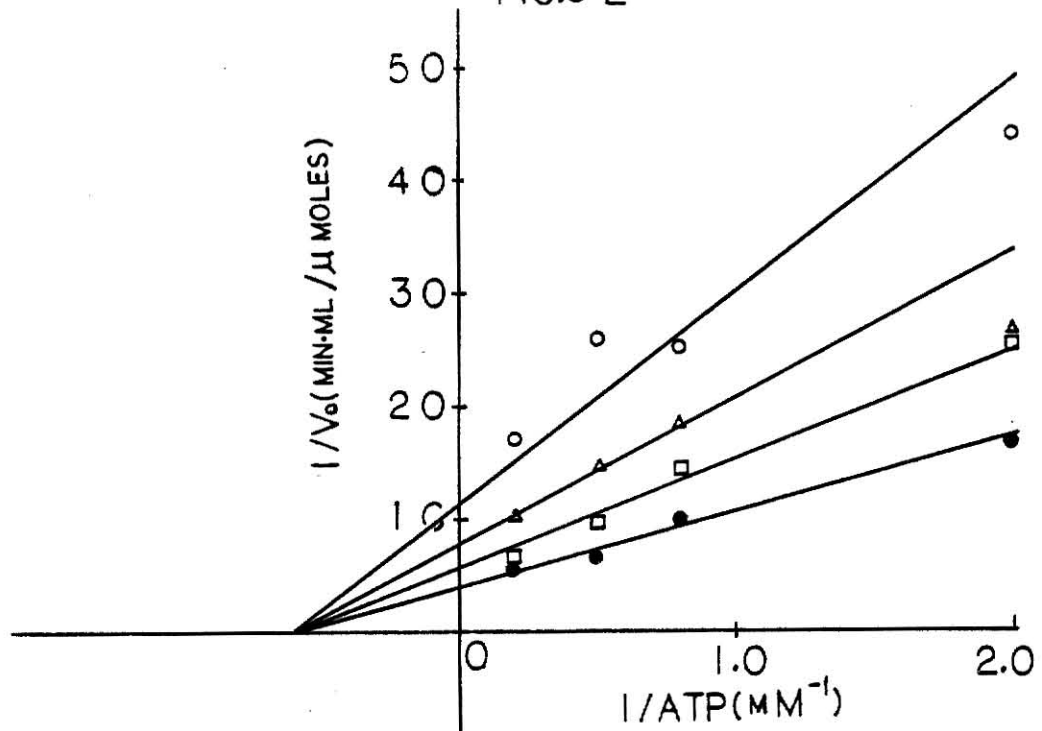
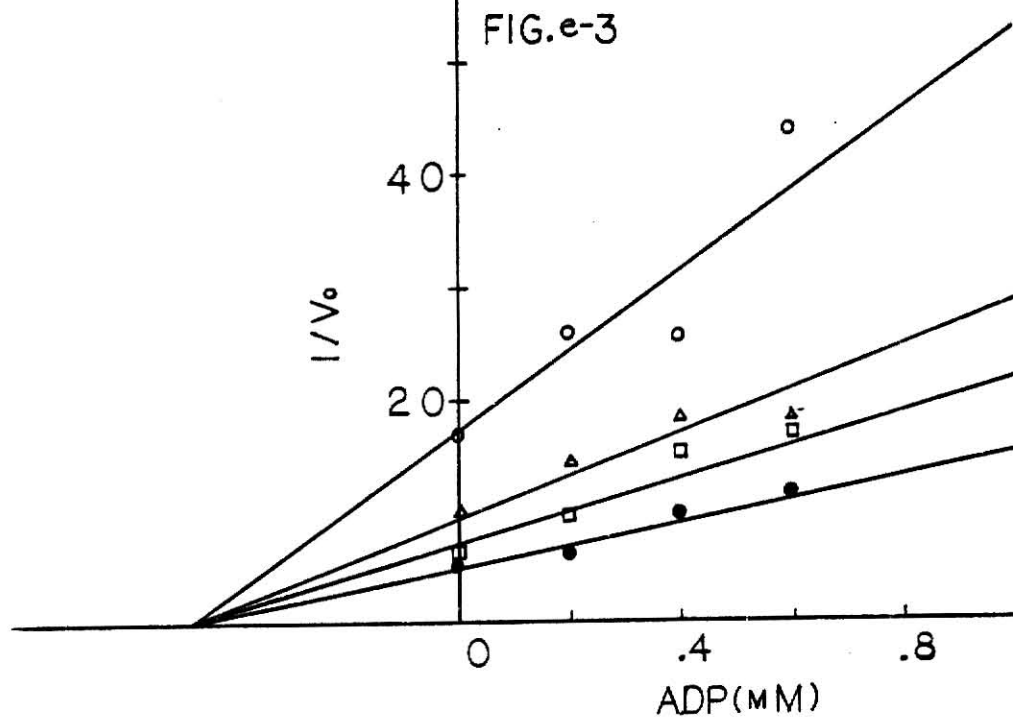


FIG.e-3



LEGEND to Figures (e-4) & (e-5)

Ratio Effect of Component 2 to Component 1 on H₂ Evolution

Figures (e-4) and (e-5) show the Lineweaver-Burk and Dixon plots from the initial velocities of H₂ evolution at different levels of ATP (0.5, 1.25, 5.0 mM) and ADP (0, 0.2, 0.4, 0.6 mM) concentrations.

Both the Lineweaver-Burk and Dixon plots appear to have similar inhibition patterns as those of ATP hydrolysis.

The calculated values of K_m and K_i are as follows:

$$K_m = 0.95 \text{ mM}$$

$$K_i = 0.95 \text{ mM.}$$

	<u>L-B plot (ADP mM)</u>	<u>Dixon plot (ATP mM)</u>
●	0.0	5.0
□	0.2	1.25
△	0.4	2.0
○	0.6	0.5

FIG.e-4

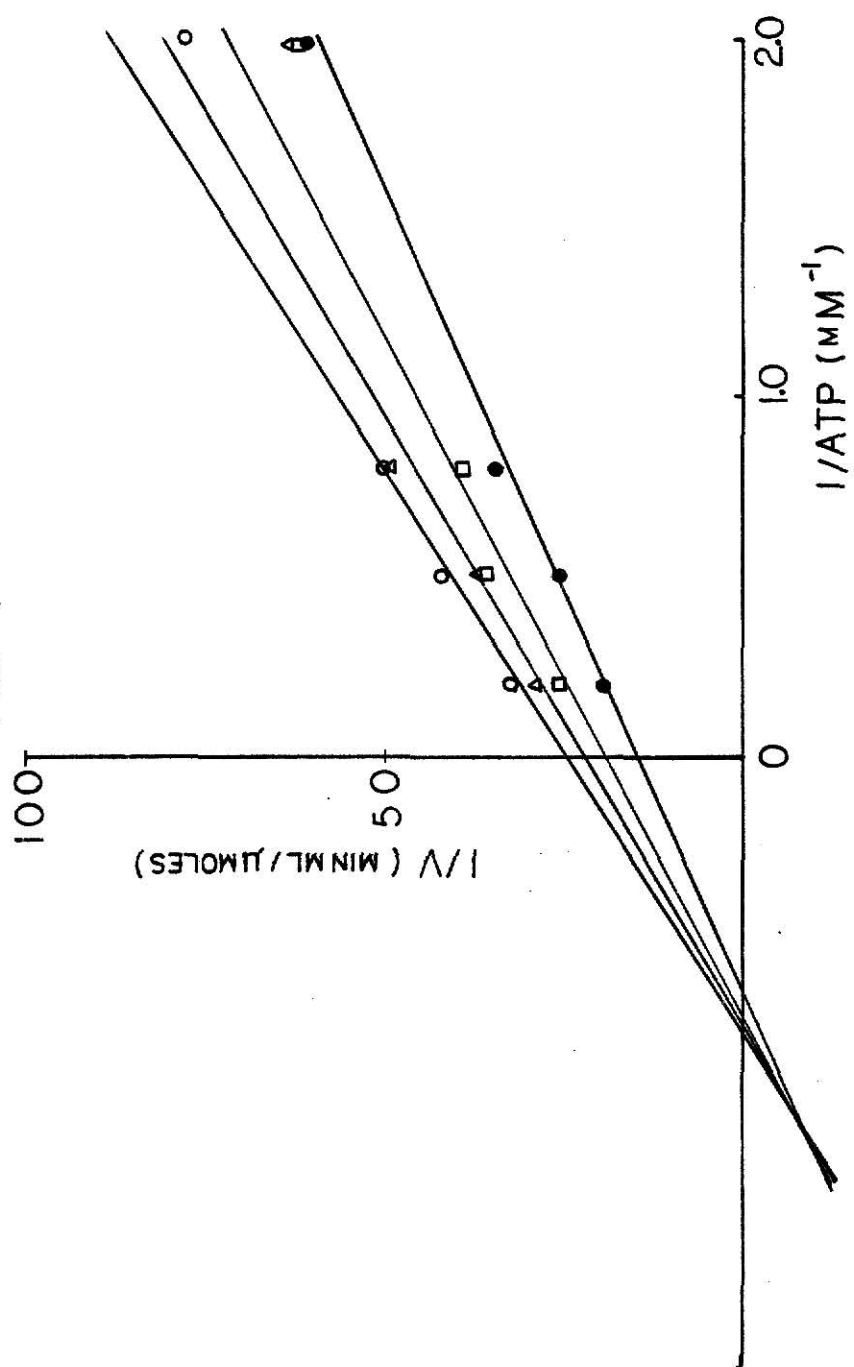
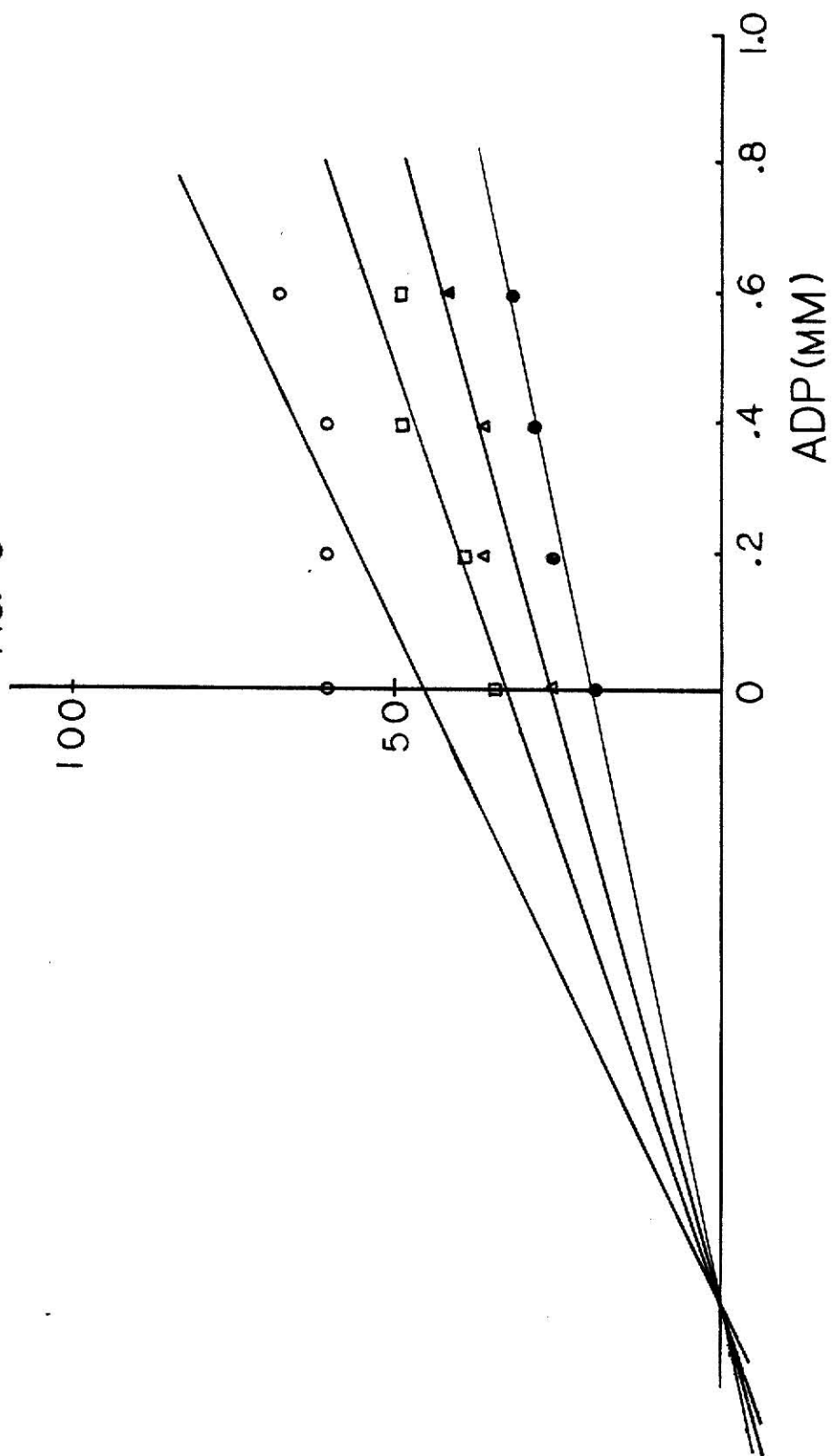


FIG.e-5



LEGEND to Figures (e-6) & (e-7)

Ratio Effect of Component 2 to Component 1 on Acetylene Reduction

The figures that follow show the Lineweaver-Burk and Dixon plots from the initial velocities of acetylene reduction at different ATP (0.5, 1.25, 5.0 mM) and ADP (0, 0.2, 0.4, 0.6 mM) concentrations.

As in the preceeding figure, both the Lineweaver-Burk and Dixon plots appear to have similar inhibition patterns as those obtained in ATP hydrolysis and H_2 evolution.

The calculated values of K_m app. and K_i are as follows:

$$K_m \text{ app.} = 1.20 \text{ mM}$$

$$K_i = 0.84 \text{ mM}$$

	<u>L-B plot (ADP mM)</u>	<u>Dixon plot (ATP mM)</u>
●	0.0	5.0
□	0.2	2.0
Δ	0.4	1.25
○	0.6	0.5

FIG. 6-6

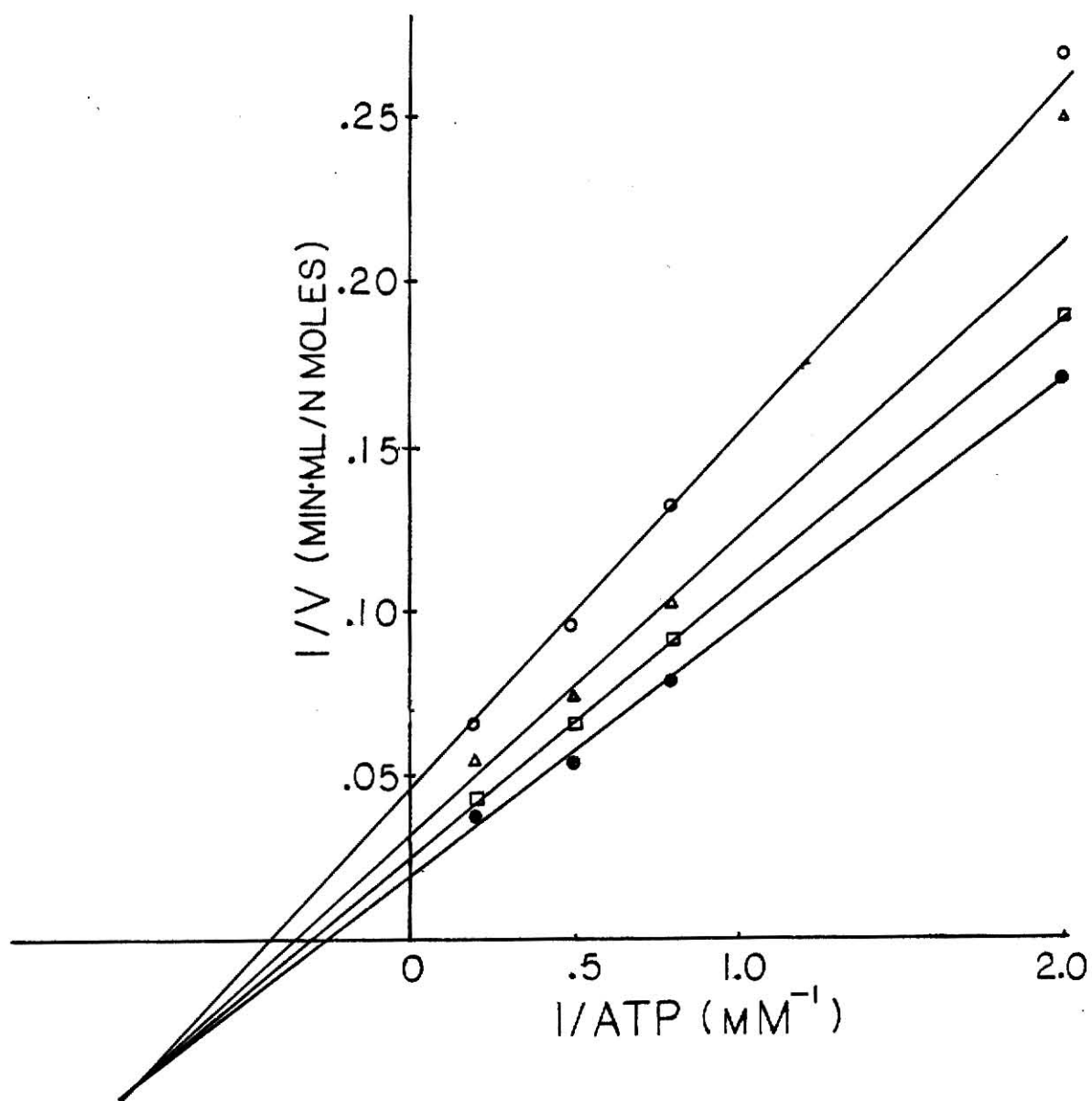
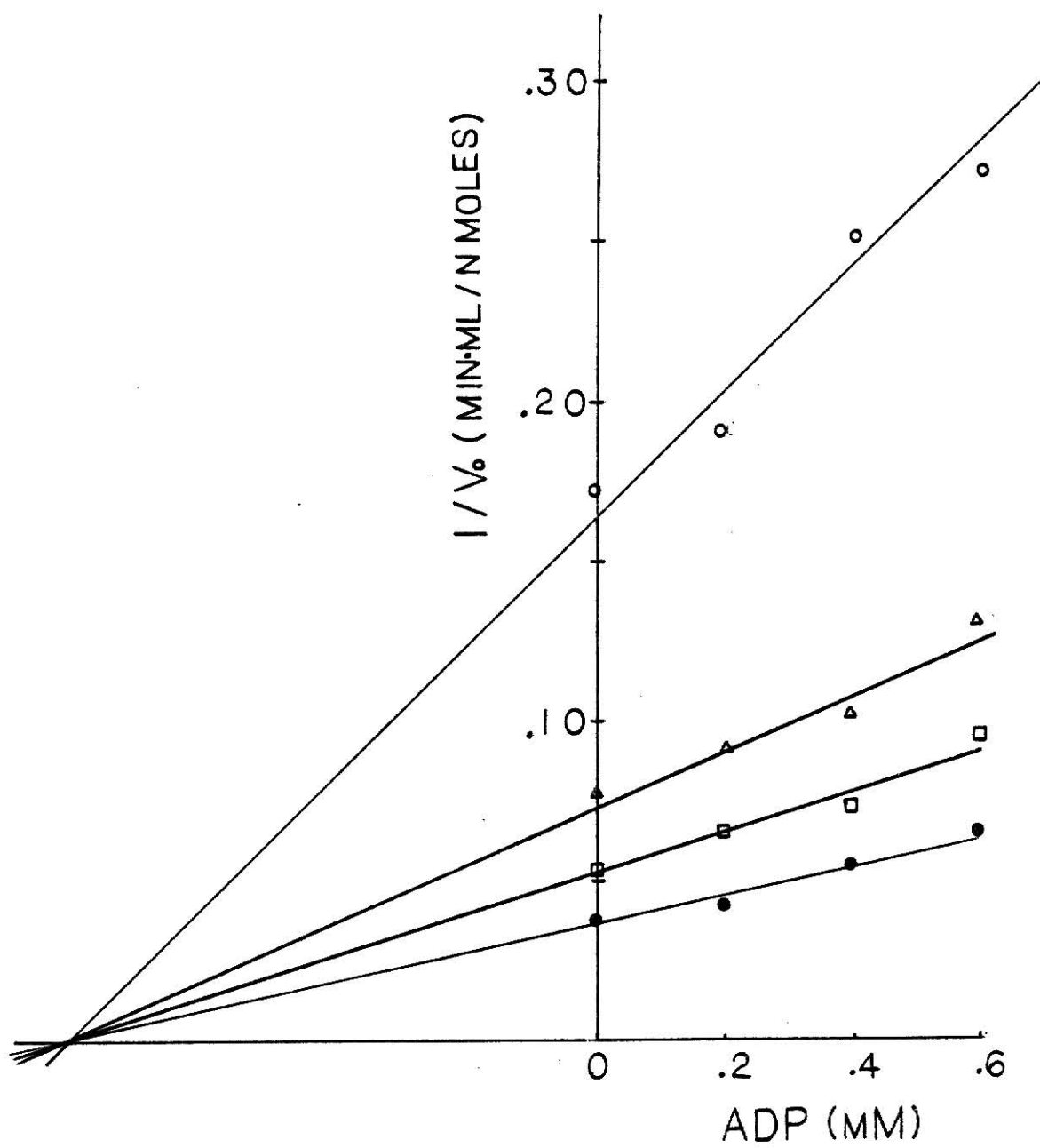


FIG.e-7



f. Determination of Initial Velocities by Rapid Chemical Quench Apparatus

The more precise initial velocities of ATP hydrolysis and acetylene reduction were obtained by means of rapid chemical quench apparatus. The reaction conditions were the same as those of enzyme studies (e). The calculated values of K_m for ATP and K_i for ADP (both are apparent K_m and K_i values) were 2.4 mM and 70 micro M respectively.

The K_m and K_i values of acetylene reduction were 9.0 mM and 30 micro M respectively. Those values of K_m 's appeared greater and those of K_i 's appeared smaller, indicating that ADP is more inhibitory, and ATP is more needed to give half V_{max} than those calculated in enzyme studies (e).

All Lineweaver-Burk plots and Dixon plots appeared to be mixed type inhibition. The 50% inhibition of ATP hydrolysis was 30 micro M of ADP ($0.5 = ADP/ADP + K_i$) and that of acetylene reduction was achieved when ADP concentration was at 70 micro M.

The calculated value of alpha (fig. f-3) was ca. 10; thus, the K_m value calculated from apparent K_m value of 2.4 mM for ATP hydrolysis gave 0.24 mM. The alpha value for acetylene reduction was likewise calculated and gave ca. 7; therefore, the K_m value of acetylene reduction was 0.2 mM. These values strongly suggest that ADP bound to Fe protein also inhibits ATP binding to free Fe protein and Fe protein-ATP complex which eventually binds one more $ATP-Mg^{2+}$ to associate with MoFe protein to transfer electron.

LEGEND to Figure (f-1)

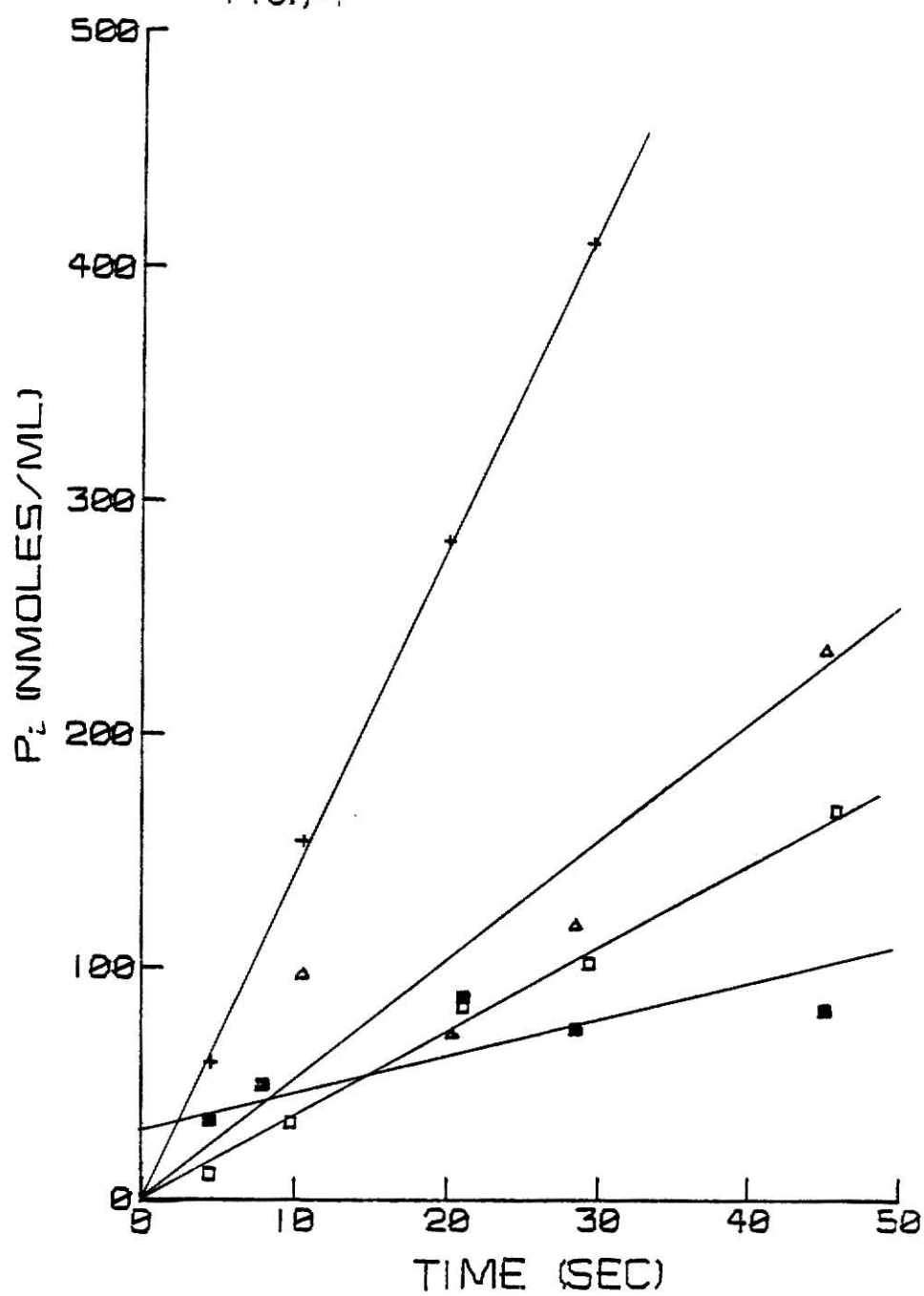
Time Course of ATP Hydrolysis Using Rapid Chemical Quench Apparatus

The ATP level in fig. (f-1) was 1 mM at different levels of ADP concentrations of 0 mM, 0.125 mM, 0.25 mM and 0.5 mM. Time intervals were 0, 5, 10, 20, 30 and 45 seconds which were measured and recorded by a modified Omniscribe Recorder to obtain precise time intervals.

It is clear that the rate of production of phosphate during ATP hydrolysis becomes more inhibited as the initial ADP concentration is set at higher levels.

■	0	mM ADP
□	0.125	"
△	0.25	"
✦	0.5	"

FIG.f-1

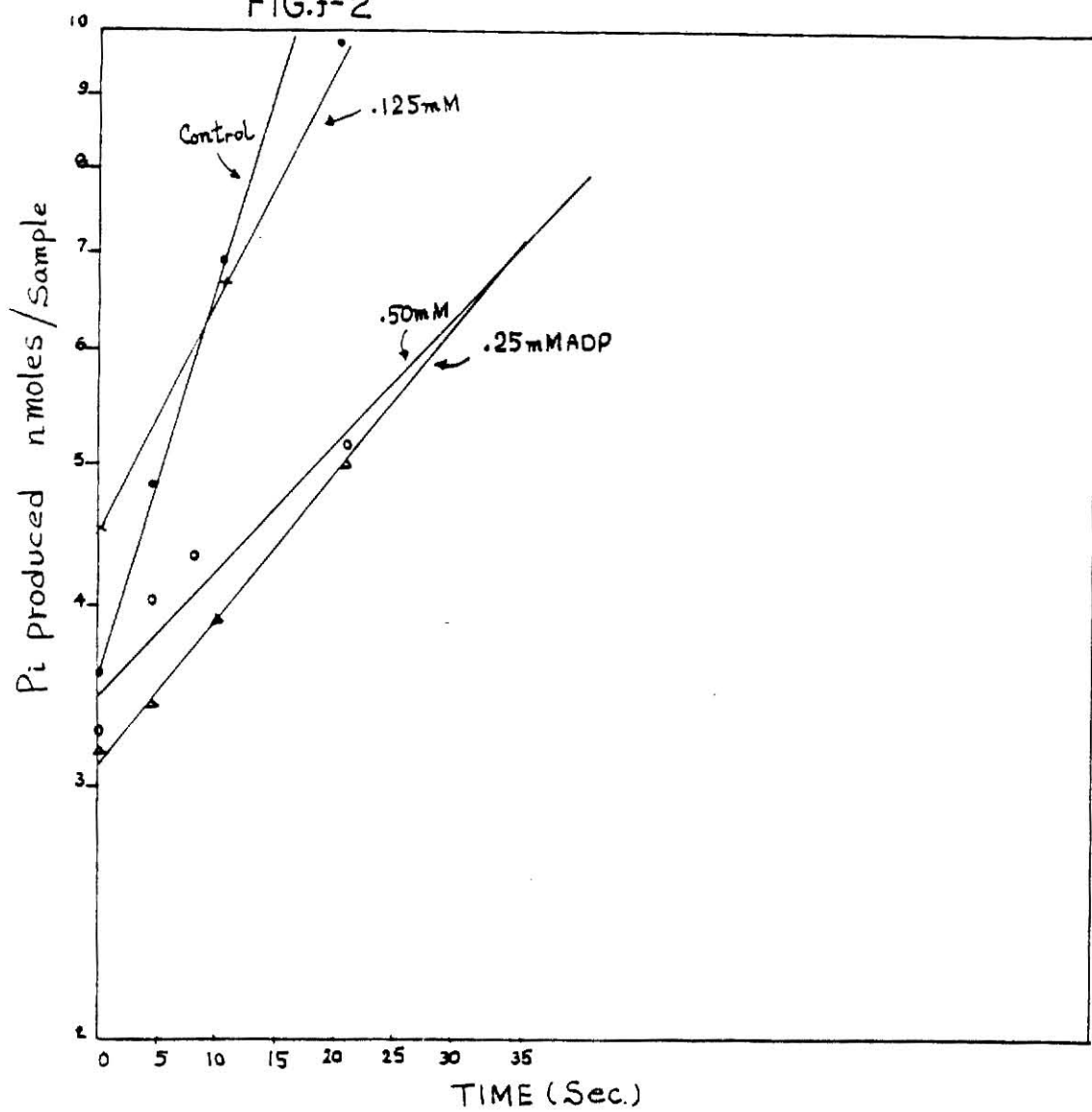


LEGEND to Figure (f-2)

Initial Velocities of ATP Hydrolysis Obtained from Rapid Chemical Quench
Apparatus

Figure (f-2) shows the replotting of the production of phosphate vs. time (from fig. f-1) on a semi-log to obtain precise initial velocities of ATP hydrolysis.

FIG.f-2



LEGEND to Table (f-1)Initial Velocities of ATP Hydrolysis at Different ATP and ADP Concentrations

The table below shows the initial velocities (in nmoles/sec. ml) of ATP hydrolysis at different ATP and ADP concentrations. ATP concentrations were varied from 1.0 mM to 5.0 mM, and ADP concentrations were varied from 0.0 mM to 0.5 mM.

Table (f-1)

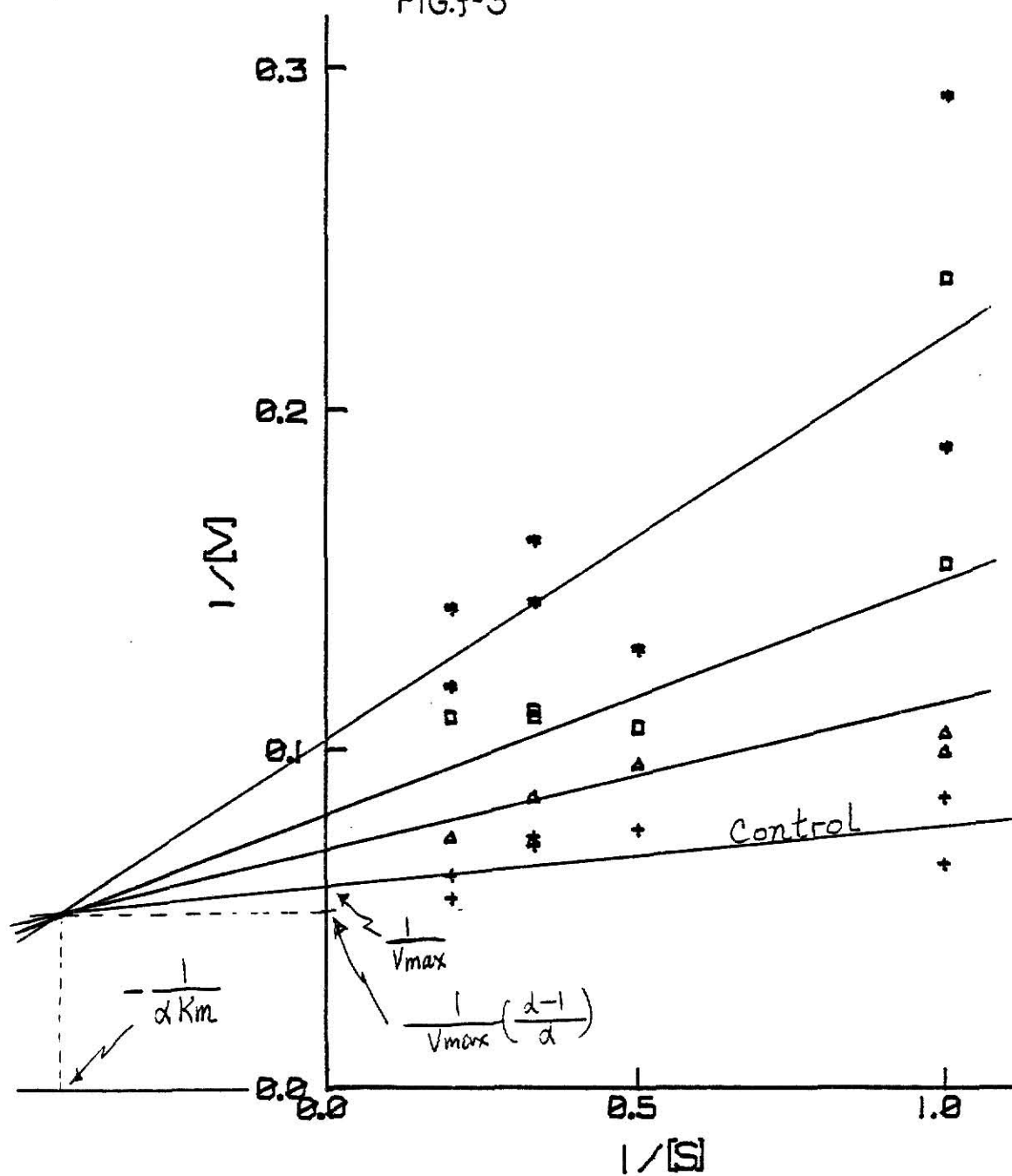
<u>ATP Concentration (mM)</u>	<u>ADP Concentration (mM)</u>			
	<u>0.0</u>	<u>0.125</u>	<u>0.25</u>	<u>0.5</u>
1.0	11.55	10.00	6.45	5.27
1.0	15.00	9.47	4.17	3.41
3.0	13.86	13.59	8.95	6.18
3.0	13.41	11.53	9.09	6.97
5.0	15.91	13.41	0.09	8.41

LEGEND to Figure (f-3)ADP Inhibition Patterns of ATP Hydrolysis as a Function of ATP and ADP Concentrations (Lineweaver-Burk Plot)

Figure (f-3) shows a Lineweaver-Burk plot of $1/V_o$ vs. $1/ATP$ which indicates a linear mixed type non-competitive inhibition pattern. ADP changes slopes and intercepts.

+ 0.0 mM ADP
Δ 0.125 "
□ 0.25 "
* 0.5 "

FIG.f-3

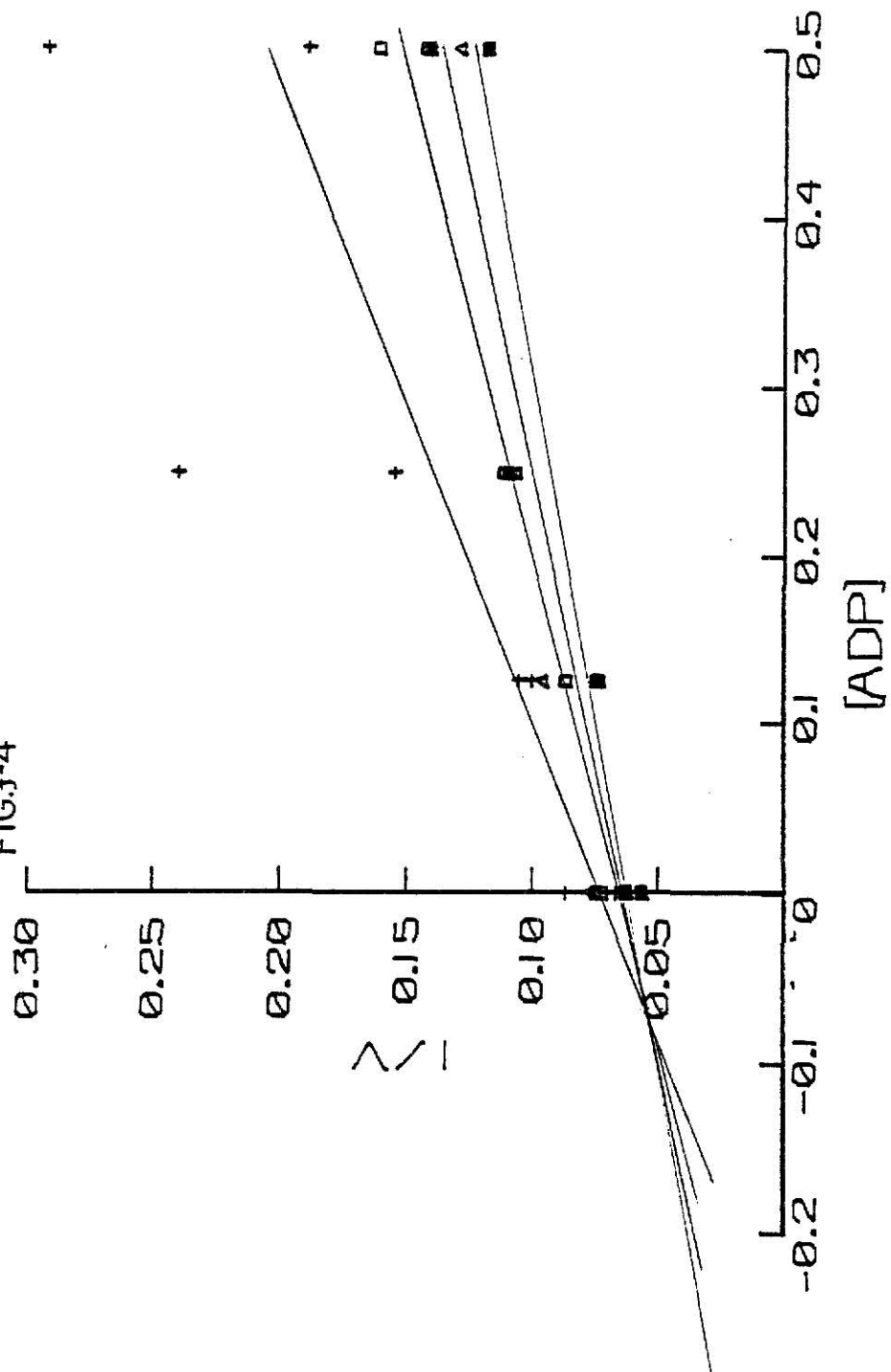


LEGEND to Figure (f-4)ADP Inhibition Patterns of ATP Hydrolysis as a Function of ATP and ADP Concentrations (Dixon Plot)

Figure (f-4) shows a Dixon plot of $1/V_0$ vs. $1/ADP$. The inhibition pattern also appears as a linear mixed type non-competitive inhibition. This suggests that the enzyme-substrate-inhibitor complex is not catalytically active and, therefore, the dissociation constant for enzyme-substrate-inhibitor complex towards enzyme and product is zero.

+		1.0 mM ATP
Δ		2.0 "
□		3.0 "
■		5.0 "

FIG.f-4



LEGEND to Figure (f-5)

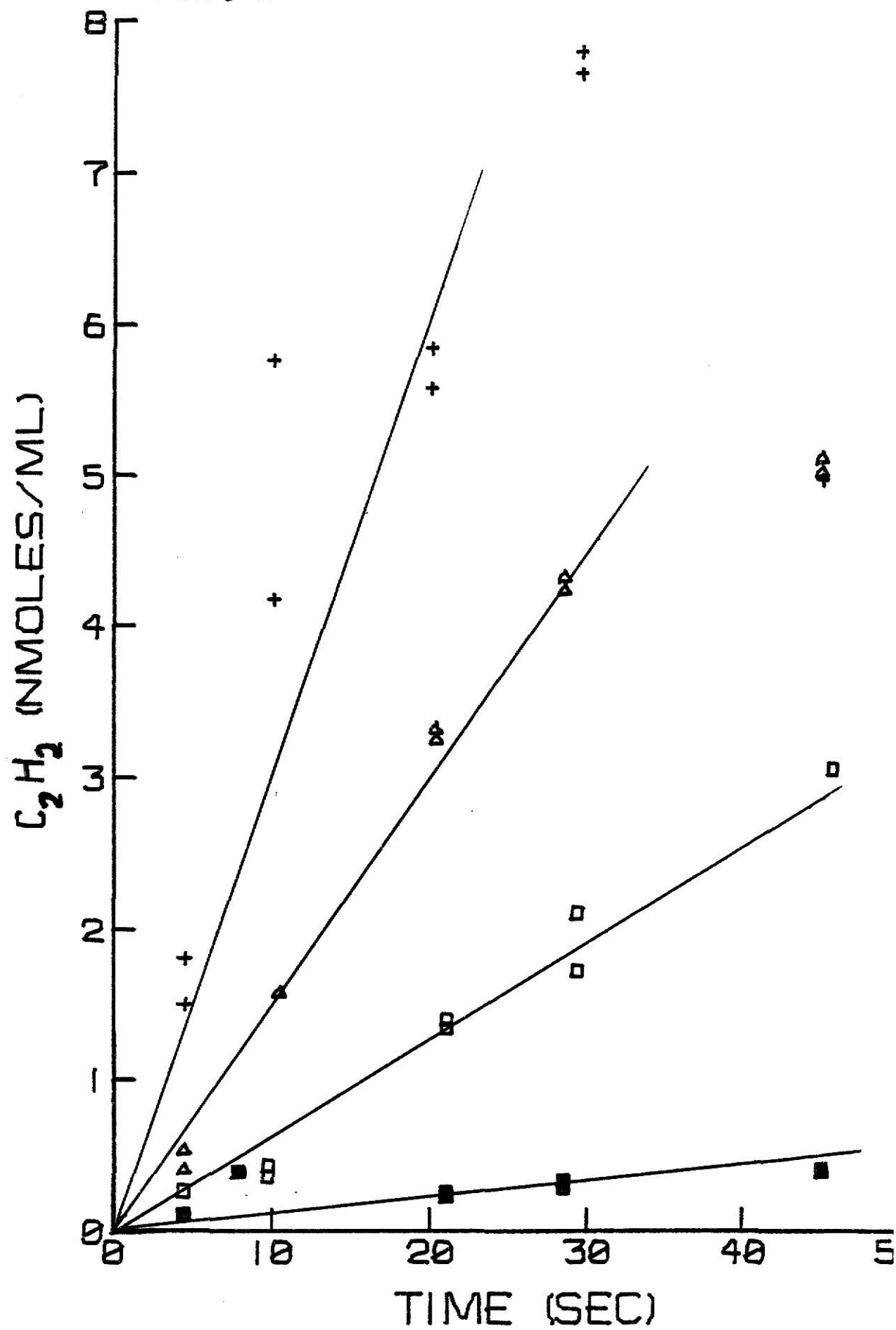
Time Course of Acetylene Reduction Using Rapid Chemical Quench Apparatus

The ATP level in fig. (f-5) was 1 mM at 0 mM, 0.125 mM, 0.25 mM & .5 mM ADP concentration levels. Time intervals were 0, 5, 10, 20, 30 and 45 seconds which were measured and recorded by a modified Omniscribe recorder to obtain precise time intervals.

It is observed that the rate of acetylene reduction is more inhibited the higher the level of initial ADP concentration.

	<u>ATP (mM)</u>	<u>ADP (mM)</u>
+	1.0	0.0
Δ	1.0	0.125
□	1.0	0.25
■	1.0	0.50

FIG.f-5



LEGEND to Table (f-2)Initial Velocities Obtained from Time Course of Acetylene Reduction

The initial velocities (in nmoles/sec. ml) of acetylene reduction were directly obtained from a time course.

The table below shows the initial velocities of acetylene reduction at different ATP concentrations (1.0, 2.0, 3.0, 5.0 mM) and different ADP concentrations (0.0, 0.125, 0.25, 0.5 mM).

Table (f-2)

<u>ATP Concentration (mM)</u>	<u>ADP Concentration (mM)</u>			
	<u>0.0</u>	<u>0.125</u>	<u>0.25</u>	<u>0.5</u>
1.0	.471	.208	.0623	.015
1.0	.360	.153	.0420	.014
2.0	.373	.212	.137	.091
3.0	.537	.364	.145	.051
5.0	.591	.415	.182	.272

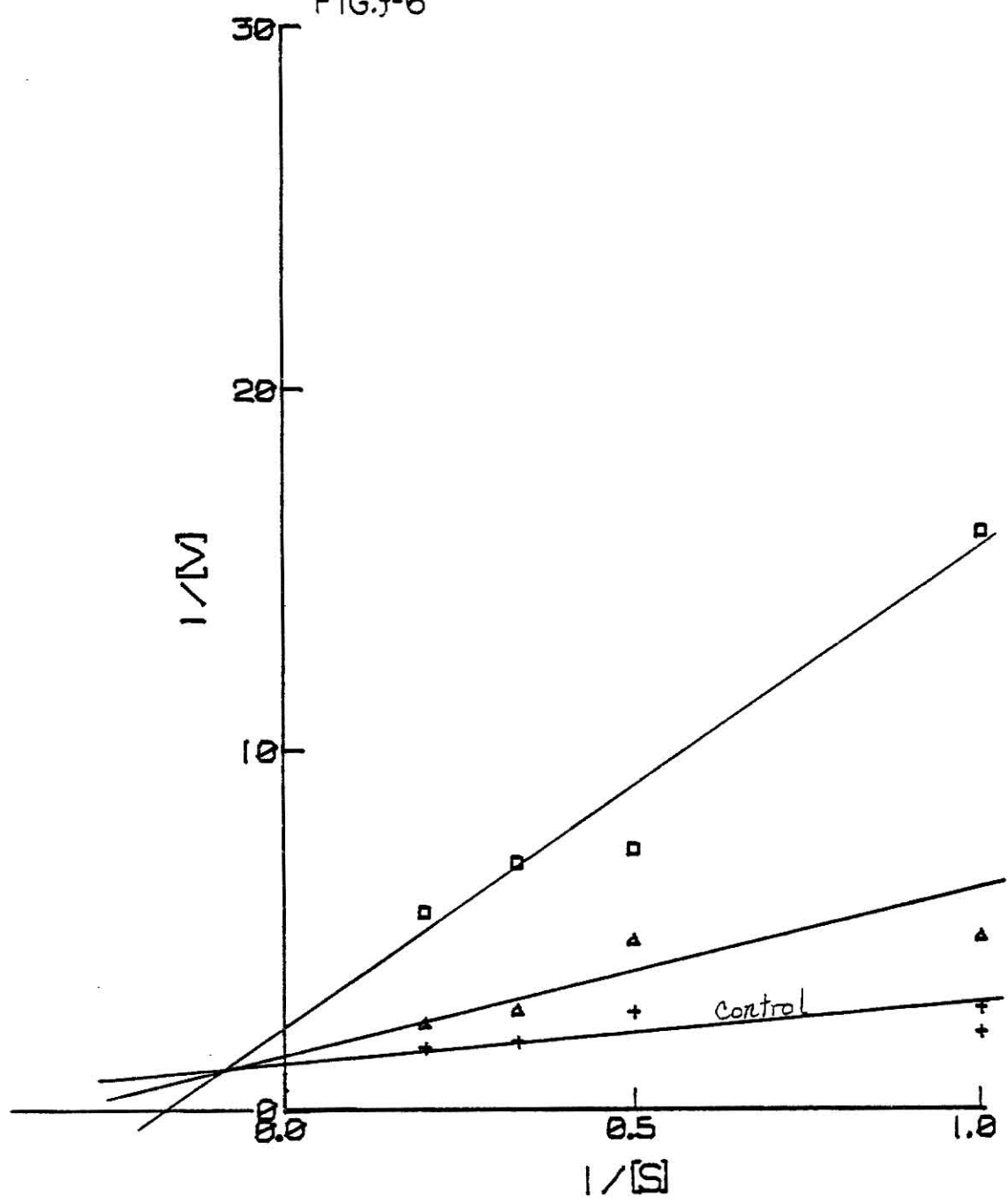
LEGEND to Figure (f-6)

ADP Inhibition Pattern of Acetylene Reduction as a function of ATP and
ADP Concentrations (Lineweaver-Burk Plot)

Figure (f-6) shows Lineweaver-Burk plot of $1/V_0$ vs. $1/ATP$ which indicates a linear mixed type non-competitive inhibition pattern. ADP changes slopes and intercepts.

+ 0.0 mM ADP
Δ 0.125 "
□ 0.25 "

FIG.f-6

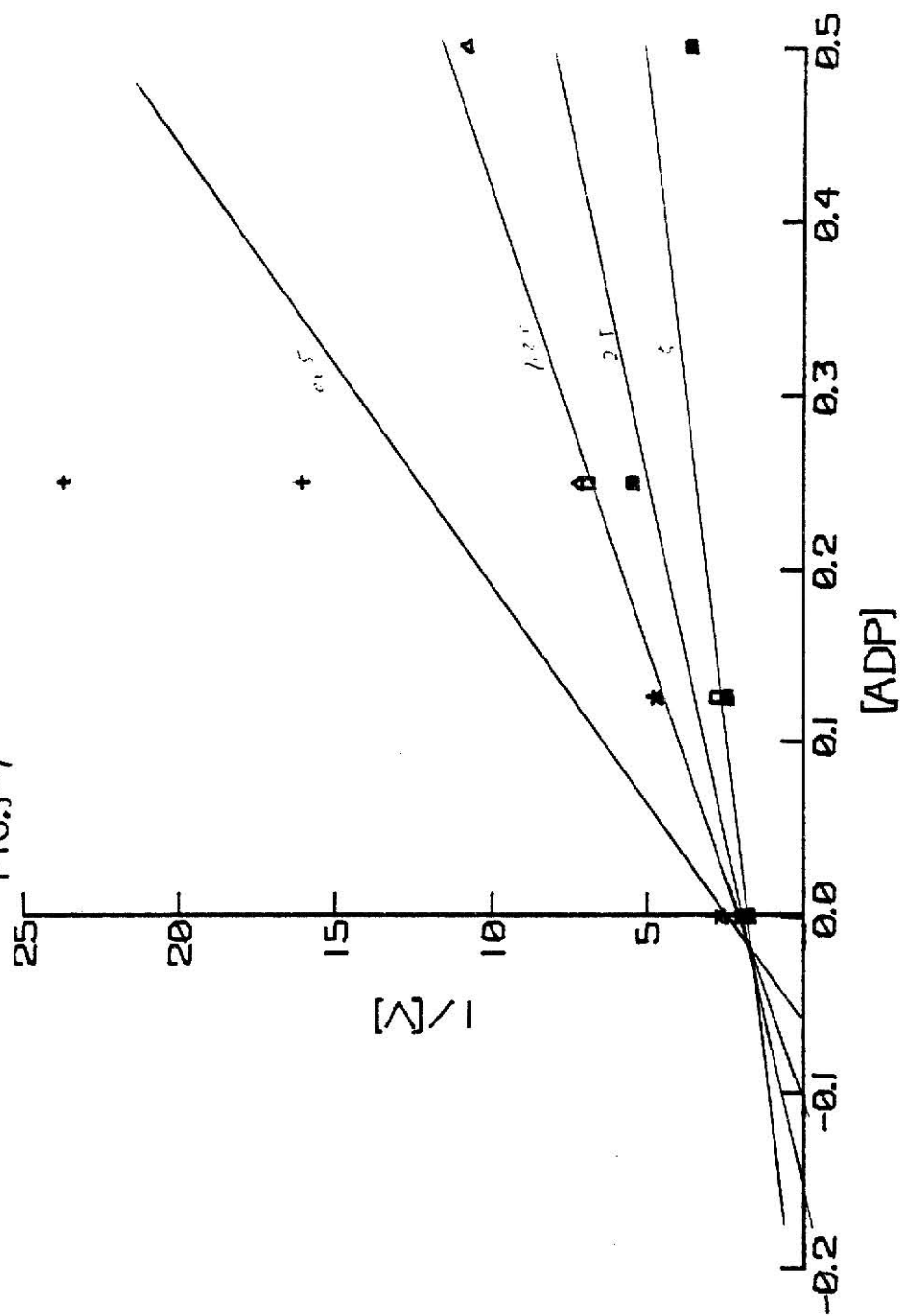


LEGEND to Figure (f-7)ADP Inhibition Pattern of Acetylene Reduction as a Function of ATP and
ADP Concentrations (Dixon Plot)

Figure (f-7) shows Dixon plot of $1/V_o$ vs. $1/ADP$. The inhibition pattern likewise appears as a linear mixed type non-competitive inhibition. This suggests that the enzyme-substrate-inhibitor complex is not catalytically active and, therefore, the dissociation constant for enzyme-substrate-inhibitor complex towards enzyme and product is zero.

■ 5.0 mM ATP
□ 3.0 "
Δ 2.0 "
+ 1.0 "

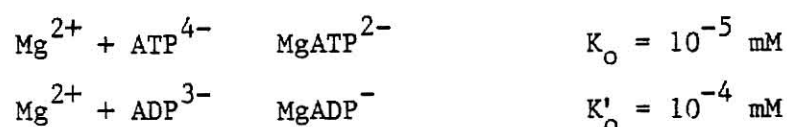
FIG.f-7



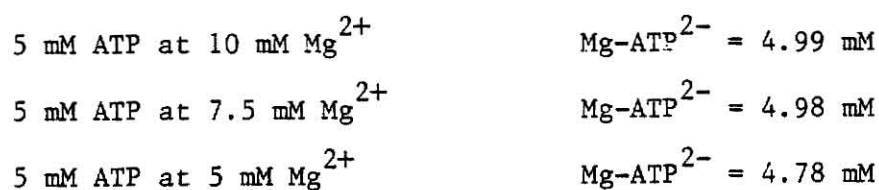
IV. CONCLUSION

Enzyme studies (a) suggests that the presence of Mg^{2+} ions is essential for nitrogenase activity, since in the absence of Mg^{2+} , no nitrogenase activity was observed. However, a higher level of Mg^{2+} seems inhibitory to nitrogenase activity in the later phase of the time course. Therefore, the product of ATP hydrolysis (i.e., ADP and/or P_i) might be an inhibitor for nitrogenase.

In the presence of Mg^{2+} , ATP and ADP can make a chelate complex with Mg^{2+} as shown below:



Under the conditions in enzyme studies (a), all ATP's make the complex with Mg^{2+} . This is shown in the calculations below.



The initial velocities seem to appear the same at different levels of Mg^{2+} concentration and at a fixed ATP concentration of 5 mM. In as much as the true substrate for Fe protein may be an Mg-ATP complex, the initial concentrations of Mg-ATP complex are the same at different levels of Mg^{2+} concentration. This result suggests that Mg^{2+} alone does not enhance or activate nitrogenase, but that it must be bound to ATP; and, only this complex can bind with Fe protein.

In enzyme studies (b), the results strongly suggest that ADP potentially decreases the initial velocities of all nitrogenase activity such

as ATP hydrolysis, hydrogen evolution and dinitrogen reduction.

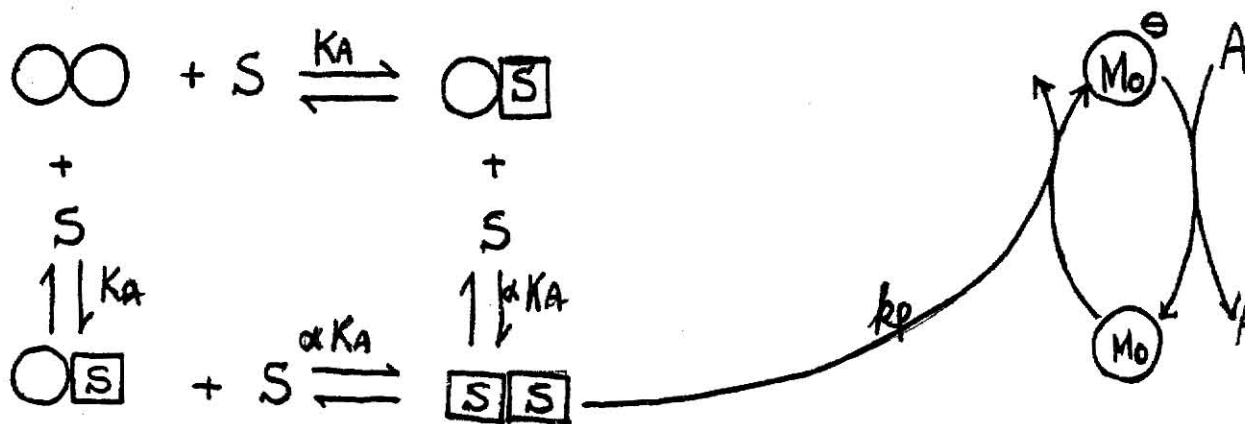
On the other hand, the results of enzyme studies (c) reveal that as ATP initial concentration increases at a fixed excess Mg^{2+} concentration of 10 mM and in the absence of ADP, the initial velocities are dependent only on the ATP concentrations.

Under the reaction conditions of enzyme studies (c), Mg^{2+} was present at a large excess. Therefore, Mg-ATP can build up to a concentration which is very high compared to Ksa (17 micro M in the case of C.pasteurium).

The following are the calculated concentrations of Mg-ATP complex at ATP concentrations ranging from 0.5 mM to 5.0 mM and with 10 mM of Mg^{2+} .

<u>ATP (mM)</u>	<u>Mg^{2+} (mM)</u>	<u>Mg-ATP (mM)</u>
0.5	10	0.50
1.25	10	1.25
2.0	10	2.0
5.0	10	4.99

The reaction mechanism might be in the scheme written below. Hence, the velocity equation can be expressed in equation (1).



where: is the Fe protein (component 2) having two identical subunits

S is Mg-ATP; P is Mg-ADP

a is a factor for Ks of $(\text{Mg-ATP})_2\text{-Fe-Protein}$ complex

kp is $(kp \gg KA, aKA)$ rate-limiting step kinetic constant under this rapid equilibrium assumption

$$v = kp (s s), \quad V_{\max} = kp (\text{Fe protein tot.})$$

$$\frac{v}{(E)t} = \frac{kp (\boxed{s} \boxed{s})}{(\boxed{s} \boxed{s}) + 2(\boxed{s} \bigcirc) + (\bigcirc \bigcirc)}$$

$$\frac{v}{V_{\max}} = \frac{\frac{s^2}{aKA}}{1 + \frac{2(s)}{KA} + \frac{(s)^2}{aKA^2}}$$

or

$$\frac{v}{V_{\max}} = \frac{\frac{(\text{Mg-ATP})^2}{aKA}}{1 + \frac{2(\text{Mg-ATP})}{KA} + \frac{(\text{Mg-ATP})^2}{aKA^2}} \quad \text{equation (1)}$$

In enzyme studies (d), both ATP and ADP concentrations were varied at fixed Mg^{2+} concentration of 10 mM to observe the influence of ADP at a fixed energy charge or ADP/ATP ratio. Since the reaction medium did not contain an energy-regenerating system such as creatine kinase-creatine phosphate system, the energy charge changed as a function of time during the reaction. The change in energy charge, however, can be ignored at the early states of reaction. The graphically obtained values of apparent K_m and K_i were 1.67 mM and 0.59 mM respectively, while those of hydrogen evolution were 1.00 mM and 0.85 mM respectively. It can be noted that those values obtained in enzyme studies (e) were almost identical to those of enzyme studies (d). The reaction conditions were the same in enzyme studies (d) and (e) except that the ratio of component 2 to component 1 was 2.5 to 1.0 in enzyme studies (d), whereas that used in enzyme studies (e) was 5.0 to 1.0 (6.0 to 1.0 in terms of mole ratio). The K_m and K_i values for ATP hydrolysis at the component ratio of 5.0/1.0 were 1.70 mM and 0.48 mM respectively; those of hydrogen evolution were 0.95 mM and 0.95 mM. Therefore, the increase in Fe protein did not change the apparent K_m or K_i values but the apparent V_{max} listed below.

	<u>2.5/1.0 ratio*</u> <u>(nmoles/min. ml)</u>	<u>5.0/1.0 ratio**</u> <u>(nmoles/min. ml)</u>
ATP Hydrolysis	92.0	140
H ₂ Evolution	10.0	50.4
Dinitrogen Reduction	10.8	-

Note: The unit nmoles/min. ml are in terms of production or utilization

of a pair of electrons.

*Data from enzyme studies (d)

**Data from enzyme studies (e)

In enzyme studies (f), it was found that: (1) both ATP hydrolysis and acetylene reduction were shown as a mixed type of inhibition pattern, and (2) the interaction value calculated from the apparent V_{max} value of Lineweaver-Burk plot came to be ca. 10. Thus, it is not only that ADP inhibits competitively with ATP, but also, ADP binding Fe protein complex enhances free ATP to bind free or mono-ATP-binding Fe proteins. The apparent K_m and K_i values for ATP hydrolysis were 2.4 mM and 70 micro M respectively. The apparent K_m and K_i values for acetylene reduction were 12.0 mM and 20 micro M respectively. These values then support the idea that nitrogenase is significantly less than optimally active if there is sufficient amount of Mg^{2+} present for ADP to exert its inhibitory effect.

The reduction of the substrates are more sensitive to ADP inhibition than is ATP hydrolysis. Similarly, substrate reduction requires more of Fe protein that donates electron to MoFe protein than does the ATP hydrolysis. Hence, it is expected that in vivo, the relative wastage of energy will be greater when energy is limiting than when it is sufficient. This could be explained by the fact that if there is sufficient energy supply but there is no nitrogen source available in a cell, the cell must utilize the energy and at the same time supply nitrogen at any rate.

The precise mechanism of this inducible enzyme nitrogenase has not been established yet. It is possible that nitrogenase could be induced only when a cell has enough supply of energy but is lacking in nitrogen source other than atmospheric dinitrogen.

The mechanism of nitrogenase catalyzed reaction as well as the mechanism of nitrogenase regulation must be more intensely investigated to

elucidate such a complicated system for a more precise knowledge on the physiological significance of nitrogen fixation.

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THE EFFECTS OF Mg^{2+} , ATP AND ADP ON ATP HYDROLYSIS AND ELECTRON
TRANSFER BY AZOTOBACTER VINELANDII NITROGENASE

by

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ABSTRACT

The ATP hydrolysis and electron transfer activities of *Azotobacter vinelandii* nitrogenase were examined by general enzyme assay method. By the use of this system, it was found that Mg^{2+} ion is essential for nitrogenase activities. In addition to the general enzyme assay method, a rapid-quenching chemical method was used to determine the precise kinetic parameters, such as apparent K_m value for ATP hydrolysis and apparent K_i value for ADP. By means of this rapid-mixing chemical quench system, it is possible to derive true initial velocities for ATP hydrolysis in the absence of a creatine phosphate-creatine kinase ATP generating system. This allows us to examine the ADP inhibition and electron transfers to reducible substrates. ADP is found to be a very potent inhibitor of nitrogenase activity with a 50% reduction of activity at an ADP/ATP ratio of 1/5. This, in turn, suggests that nitrogenase is significantly less than optimally active in vivo if the estimated value of energy charge of the intact cell (0.8) is correct and there is sufficient Mg^{2+} ions present for the ADP to exert its inhibitory effect.