Hsiang, M. W., and Bright, H. J. (1967b), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 26*, 605.
Hsiang, M. W., and Bright, H. J. (1969), *Methods Enzymol. 13*, 347.
Hughes, T. R., and Klotz, I. M. (1956), *Methods Biochem. Anal. 3*, 265.
Luz, Z., and Meiboom, S. (1964), *J. Chem. Phys. 40*, 2686.
Mildvan, A. S. (1970), Enzymes, New York, N. Y., Academic, 3rd ed (in press).
Mildvan, A. S., and Cohn, M. (1963), *Biochemistry 2*, 910.
Mildvan, A. S., and Cohn, M. (1970), *Enzymology 32* (in press).
Mildvan, A. S., and Scrutton, M. C. (1967), *Biochemistry 6*, 2978.
Williams, V. R., and Libano, W. Y. (1966), *Biochim. Biophys. Acta 118*, 144.

Kinetic Studies of the Nitrogenase-Catalyzed Hydrogen Evolution and Nitrogen Reduction Reactions*

Richard Silverstein† and W. A. Bulen‡

ABSTRACT: The nitrogenase enzyme from *Azotobacter vinelandii* can be considered as a complex of an Fe–Mo protein and an Fe protein that catalyzes H_2 evolution, the reduction of N_2 to NH_3, and the reduction of other substrates. All reactions are ATP dependent, releasing inorganic phosphate. Initial rates of H_2 evolution are reported as a function of both ATP and nitrogenase concentration. ATP rate profiles are sigmoidal. Nitrogenase profiles are concave upward becoming linear at higher concentrations. Computer simulation was used to correlate the rate and product data with a proposed reaction pathway involving: (1) a dynamic equilibrium between an associated enzyme species and its components, (2) the subsequent binding of two ATP molecules to the associated enzyme complex, and (3) breakdown of the resulting intermediate along two routes to yield products. ATP initial rate profiles for H_2 evolution in reactions conducted under N_2 were found to be hyperbolic, with the relative amounts of NH_3 to H_2 formation increasing at high ATP levels. The total number of electrons transferred to form products remained the same under N_2 as under argon at each ATP concentration studied. Introduction of the equilibrium constants for component association and the rate constants obtained in the treatment of H_2 evolution data provided, by computer calculation, an approximation of the concentration of intermediate reactive states of the enzyme leading to H_2 evolution and ammonia formation, respectively, as a function of ATP concentration.

Studies with the nitrogenase enzyme from *Azotobacter vinelandii* (Bulen and LeComte, 1966), *Clostridium pasteurianium* (Mortenson *et al.*, 1967), and more recently a number of other sources have demonstrated that the enzymatic activity results from the cooperative action of two protein components, an Fe–Mo protein and an Fe protein. Since neither protein has yet been shown to possess any catalytic activity alone, the components could be viewed as dissimilar subunits where the term applies to the dissociated species which may or may not be individually composed of monomeric units.

For activity the nitrogenase complex requires a source of electrons, which can be supplied *via* $Na_2S_2O_4$ (Bulen *et al.*, 1965) and ATP, which is hydrolyzed to ADP and P_i (Mortenson, 1964; D'Eustachio and Hardy, 1964; Bulen *et al.*, 1964).

A considerable amount of additional information has been accumulated relating to the reactions catalyzed by nitrogenase. In addition to N_2, it will also catalyze the reduction of C_2H_2, N_2O, N_3^-, HCN, CH_3NC, and certain analogs of some of these substrates. ATP hydrolysis studies (at 5mM ATP) have shown that (1) the rate of hydrolysis is constant with a given enzyme preparation and (2) the ratio of ATP hydrolyzed to electrons transferred to form product is independent of the substrate being reduced but exhibits a temperature dependence with ATP:$2e^-$ values ranging from 4.3 at 20° to 5.8 at 40° with values approaching 5 at 30° (Bulen and LeComte, 1966; Hadfield and Bulen, 1969). Kinetic data associated with these reactivities are, however, quite meager and it is the purpose of this report to contribute additional kinetic information obtained with the nitrogenase complex from *A. vinelandii*. Hardy *et al.* (1968) observed that C_2H_2 reduction was related to the enzyme concentration of a heated extract of N_2-grown *A. vinelandii* in a sigmoidal fashion and Moustafa and Mortenson (1967) reported that the initial rate of acetylene reduction by partially purified nitrogenase components from *C. pasteurianium* components was affected by ATP concentration.

In the absence of N_2 (or other reducible substrate) nitro-

* Contribution No. 386 of the Charles F. Kettering Research Laboratory, Yellow Springs, Ohio 45387. *Received May 6, 1970.* A preliminary report of this research was presented before the 158th National Meeting of the American Chemical Society, Sept 1969, New York, N. Y.

† Present address: Department of Biochemistry, University of Kansas Medical Center, Kansas City, Kansas 66103.
‡ To whom to address correspondence.

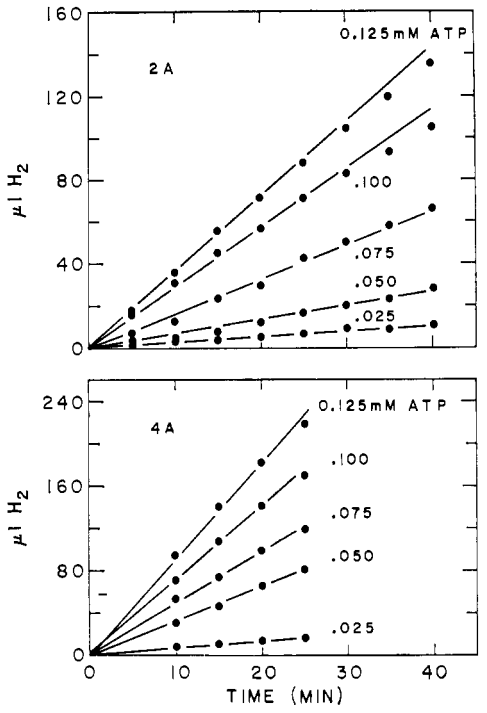

FIGURE 1: Initial rate data of ATP-dependent H_2 evolution. Assay reaction mixtures contained 50 mM TES buffer, pH 7.25, 50 mM creatine phosphate, 0.25 mg/ml of creatine kinase, 5 mM $MgCl_2$, 20 mM $Na_2S_2O_4$, and the nitrogenase complex at concentrations 2A = 0.53 mg/ml and 4A = 1.06 mg/ml. ATP was added as indicated. Incubations were: 4-ml reactions at 30° with shaking (120 oscillations/min); atm, Ar.

genase catalyzes an ATP-dependent H_2 evolution reaction (Bulen *et al.*, 1965; Burns and Bulen, 1965) which also requires both components of the complex (Bulen and LeComte, 1966). This is the least complicated reaction in that all electrons made available *via* the ATP reaction appear in the same product, H_2. It is conveniently monitored manometrically providing a simple system for kinetic analysis. This report describes a kinetic study of the H_2 evolution reaction and suggests a simplified reaction sequence consistent with the kinetic observations and data examined with the computer program REMECH, of DeTar and DeTar (1966, 1968). Also reported are the effects of ATP concentration on the rate and the ratio of products (NH_3, H_2) formed by reactions conducted under N_2.

Experimental Section

N-Tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid[1] was synthesized essentially as described by Good *et al.* (1966) or purchased from Calbiochem. Creatine phosphate was synthesized in our laboratory by Dr. J. L. Corbin. Stock ATP solutions (0.1 M) were prepared fresh every 2 weeks and stored frozen. Other reagents were prepared and H_2 evolution reactions were conducted essentially as previously described (Bulen and LeComte, 1966). Inorganic phosphate was measured by a previously described adaptation of the method of Furchgott and de Gubareff (1956). Protein was determined with the Biuret reagent of Gornall *et al.* (1949).

H_2 evolution from reactions conducted under N_2 were corrected for N_2 uptake. NH_3 was separated by microdiffusion and determined colorimetrically with Nessler's reagent (Bulen *et al.*, 1965). Time corrections for pipetting aliquots from the reaction mixtures of a given series were made individually (0.5–2.5 min).

The nitrogenase complex was separated from *A. vinelandii* extracts by the protamine sulfate technique followed by polyacrylamide gel fractionation (Bulen and LeComte, 1966) substituting Bio-Gel P-150 for the P-200 column previously described. All steps were performed under strictly anaerobic conditions and all buffer solutions contained 0.1 mg of dithiothreitol per ml. Additional purification was achieved either by $MgCl_2$ precipitation (twice) of the P-150 fraction or, later, by separation of the complex from the protamine sulfate fraction by $MgCl_2$ precipitation (twice) followed by chromatography on Sephadex G-150. In the latter (preferred) procedure the protamine fraction was centrifuged at 205,000*g* for 30 min. The clear supernatant solution was subjected to $MgCl_2$ fractionation to remove low molecular weight contaminants. Sufficient 2 M $MgCl_2$ in 10 mM TES, pH 7.1, was added to give a final concentration of 20 mM and the preparation centrifuged at 30,000*g* for 15 min. The supernatant solution was decanted and centrifuged at 205,000*g* for 2 hr. Pellets were resuspended in 20 mM TES, pH 6.8, and centrifuged at 60,000*g* for 10 min. The clear supernatant fraction containing *ca.* 20 mg of protein/ml was made 10 mM with $MgCl_2$. Centrifugation at 20,000*g* for 15 min followed by centrifugation of the supernatant solution at 205,000*g* for 2 hr again provided pellets of the active complex. These were suspended in TES buffer, pH 6.8, and centrifuged at 60,000*g* for 10 min to give a clear supernatant fraction (C42II) containing *ca.* 25 mg of protein/ml. Large molecular weight contaminants were removed by adding the C42II material (*ca.* 25 ml) to a 4 × 20 cm column of Sephadex G-150 equilibrated with water and eluted with 20 mM TES, pH 7.2. All material not retained by the gel was discarded. The nitrogenase complex started to elute after *ca.* 140 ml of total eluate had been collected and was collected in *ca.* 80 ml. For storage the G-150 fractions, subsequently referred to as the purified nitrogenase complex (PNC), were concentrated by ultrafiltration at room temperature to 20–25 mg of protein/ml, made 5 mM with $MgCl_2$, and stored under Ar in ice. These preparations were stable for periods up to 1 month but lost activity if exposed to air. Specific activities ranged from 85 to 100 nmoles of N_2 reduced per min per mg of protein depending upon the time lapse between purification steps.

The preparations were examined periodically in the ultracentrifuge using *ca.* 10 mg of protein/ml, previously desalted on Bio-Gel P-2 columns, and the purity estimated by the symmetry of the observed refractive index gradients and the area under the curves. Contaminants, if detected, accounted for *ca.* 5% of the total area under the curves. The absence of a cytochrome-type spectra is also a useful measure of purity. When subjected to the DEAE column-fractionation procedure (Bulen and LeComte, 1966), all protein added was retained by the column and elution showed the presence of a pink Fe protein in addition to the Fe–Mo protein (I) and the Fe protein (II) required for nitrogenase activity using $Na_2S_2O_4$ as the electron donor. Additional details of the purification and properties of the complex will appear elsewhere (Bulen and Le-

[1] The abbreviation used is: TES, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid.

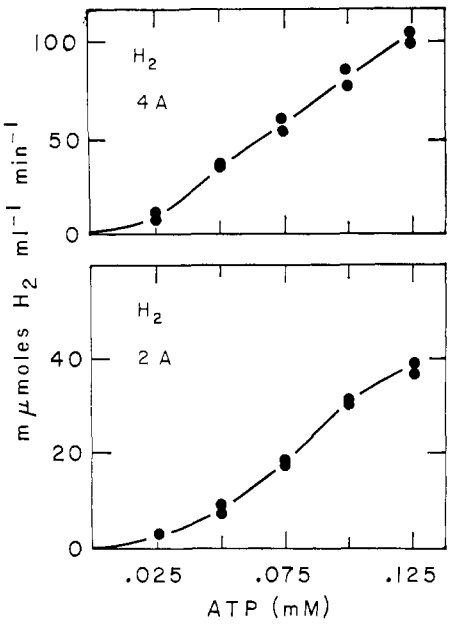

FIGURE 2: ATP rate profiles of H_2 evolution. Rates are from the data of Figure 1.

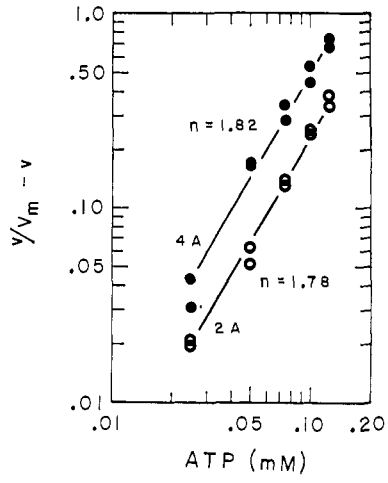

FIGURE 3: Hill treatment of the ATP rate data showing interaction coefficients n. Slopes were determined by the least-squares method.

Comte, 1970). The ability to store the PNC preparations in ice, the lack of the need to quick-freeze and store the Fe protein in liquid nitrogen and the absence of the complications induced by recombination requirements made the use of the complex preferable to the use of the separated components for the experiments described here.

Correlation of experimental data with the proposed reaction pathway was accomplished through the application of REMECH, a computer program written by DeTar and DeTar (1966, 1968). Calculations were performed on the IBM 7094 or 6400 computer.

Results

H_2 evolved from reaction mixtures containing 0.025–0.125 mM ATP was sufficiently proportional with time to allow reliable detemination of initial rates (Figure 1). It was further established that doubling the creatine kinase concentration of the generating system gave no rate enhancement at all ATP and nitrogenase concentrations used. From equilibrium data of the creatine phosphate–creatine kinase reaction (Nihei *et al.*, 1961), the steady-state concentration of ADP was calculated for all reactions to be less than 1% that of ATP.[2]

Plots of H_2 evolved *vs.* ATP concentration yielded sigmoid curves at low ATP concentrations (Figure 2). Application of the Hill equation

$$\log [v/(V_{max} - v)] = n \log (S) - \log K \qquad (1)$$

gave interaction coefficients n (Monod *et al.*, 1963; Atkinson *et al.*, 1965; and other references therein) for these data of 1.78 and 1.82, respectively (Figure 3). n and K are empirical constants; v and V_{max} are initial velocities at given concentrations of S and at saturation, respectively. The V_{max} values used for the calculations were from rates obtained with 5 mM ATP and are viewed as minimal since the rates decreased with time in the final minutes of the reaction period at this ATP level. Values of n calculated from other sets of data varied between 1.7 and 1.9. Calculated V_{max} values were sufficiently great compared with all corresponding v values so as to make n virtually insensitive to V_{max}.

P_i formation was determined for reactions at the higher nitrogenase concentration (4A of Figure 1). The P_i:H_2 values at 30° were independent of ATP concentration and centered around 5 (Table I).

ATP rate profiles of H_2 evolution and ammonia formation from reactions conducted under N_2 are shown in Figure 4. Rates of H_2 evolution, in all reactions were linear for the 30-min reaction time. It is seen that the profiles for H_2 evolution are hyperbolic whereas the corresponding NH_3 profiles are sigmoidal. Thus the ratio of NH_3 to H_2 increases with increasing ATP concentrations. Despite larger experimental errors at the lower ATP levels, repeated experiments yielded similar results.

TABLE I: Effect of ATP on P_i:H_2 Product Ratio.[a]

ATP (mM)	P_i (μmoles)	H_2 (μmoles)	P_i:H_2
0.05	22.8	4.20	5.4
0.075	30.4	6.24	4.9
0.10	44.0	9.16	4.8
0.125	58.0	11.1	5.2
5.0	69.6	13.9	5.0

[a] Data are products of the reactions at 30 min for the reaction mixtures of Figure 1 (4A) using nitrogenase at 1.06 mg/ml. Reaction volumes were 4 ml, except for the 5 mM ATP reactions which were 2 ml.

[2] Equilibrium conditions require an increase in the concentration of ADP as the ratio of creatine to creatine phosphate increases during the course of the reaction. This will eventually contribute to a tapering off of the rate.

TABLE II: Product Formation and Electron Utilization as a Function of ATP Concentration.[a]

ATP (mM)	N_2				Ar
	NH_3 (μmoles)	H_2 (μmoles)	NH_3:H_2	$2e^-$ (μmoles)[b]	$H_2(2e^-)$ (μmoles)
0.025	0.19	1.47	0.13	1.76	1.96
0.050	1.40	3.69	0.38	5.79	5.85
0.075	3.11	5.84	0.53	10.51	10.49
0.100	4.48	7.42	0.61	14.14	14.39

[a] Reaction mixtures and conditions as given for Figure 1. Nitrogenase complex 1.01 mg/ml. Incubation 30 min at 30°. [b] $2e^- = (1.5) \times \mu$moles of $NH_3 + \mu$moles of H_2.

Table II contains reduced product data from equivalent reactions conducted under argon and under N_2. Despite changes in the NH_3 to H_2 product ratio with increasing ATP concentration, the total number of electron pairs ($2e^-$) transferred to product was the same for each atmosphere at each ATP concentration.

Initial reaction rates were not proportional to enzyme concentration under certain conditions. With sufficiently low concentrations of both ATP and nitrogenase, initial rates were greater than first order with respect to nitrogenase concentration yielding curves that were concave upward (Figure 5). The rate curves become increasingly concave upward in going from 0.25 to 0.075 mM ATP, but even at 1 mM ATP curvature was observed when nitrogenase was maintained within a sufficiently low concentration range.

The possibility that concave upward nitrogenase profiles resulted from variable inactivation of the enzyme by contaminants in one of the reagents was excluded by showing that individual variations of two- to fourfold in the concentration of all other reagents ($MgCl_2$, $Na_2S_2O_4$, creatine phosphate, creatine kinase, TES) produced no change in reaction rate or degree of profile curvature. In addition, concave upward nitrogenase profiles were obtained with each of several enzyme preparations used.

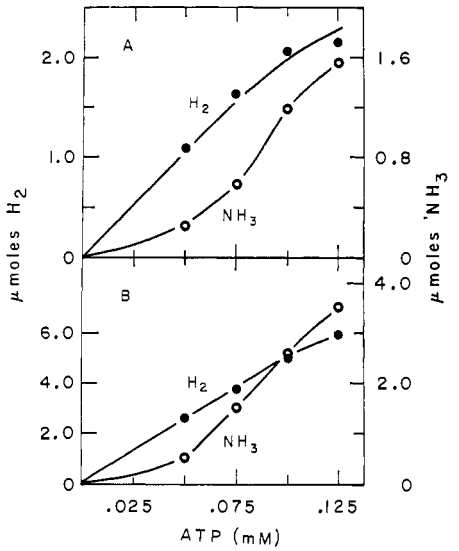

FIGURE 4: ATP rate profiles of H_2 evolution and NH_3 formation under N_2. Reaction mixtures and conditions as given for Figure 1. Nitrogenase concentrations were: A = 0.59 mg/ml, B = 1.06 mg/ml (different preparations); atm, N_2; reaction time, 30 min.

Discussion

The derivation of rate equations for the study of enzyme mechanisms commonly considers the enzyme as a monomeric species, E. While this consideration is no doubt valid for a large majority of enzymes, it is not designed to include systems in which, during catalysis, E may undergo rapid and reversible dissociation or association of functionally required components. Since the degree of association of a system in dynamic equilibrium is concentration dependent, it should not be surprising to find initial rate profiles nonlinear with enzyme concentration but dependent on the relative activities of the equilibrating species. Nitrogenase is being considered as a complex of dissimilar components which, for the purpose of this discussion, can be considered as dissociable dissimilar subunits, neither being catalytically active in the absence of the other. In this context the observed rate profiles are believed to be the result of subunit interaction. It is noteworthy for comparative purposes, that the specific activity of bovine liver

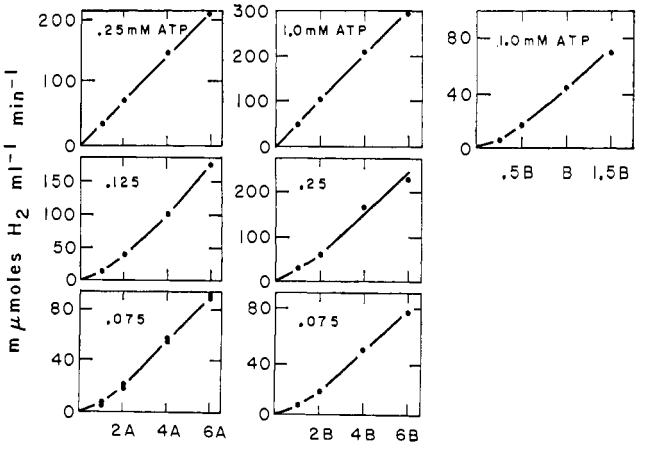

FIGURE 5: Nitrogenase rate profiles of H_2 evolution and their dependence on ATP concentration. Reaction mixtures and conditions as given for Figure 1 using the indicated ATP concentrations. Nitrogenase concentrations are given in multiples of A and B for two respective preparations where A = 0.265 mg/ml and B = 0.206 mg/ml.

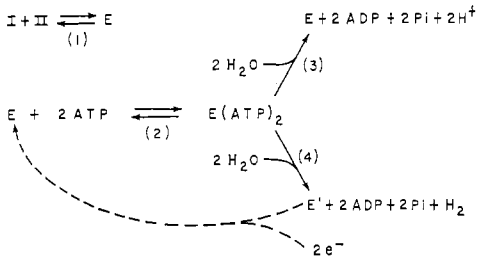

FIGURE 6: Proposed reaction pathway for ATP-dependent H_2 evolution in the absence of added substrate. For explanation, see text.

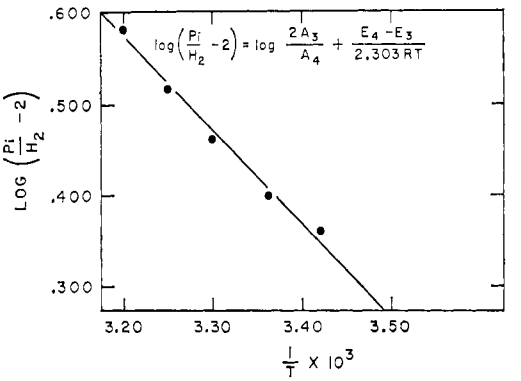

FIGURE 7: Correlation of temperature-dependent P_i:H_2 data (Hadfield and Bulen, 1969) with steps 3 and 4 of Figure 6 by application of the Arrhenius equation. For detailed explanation, see text.

glutamate dehydrogenase, a reversibly dissociating enzyme, gives concentration-dependent specific activity values (Grisolia *et al.*, 1962). Its rate of dissociation is very rapid, with an estimated half-life of 50 msec (Fisher and Bard, 1970). The specific activity of the dissociating enzyme glycogen phosphorylase *a* is also concentration dependent (Huang and Graves, 1970, and references therein). Each of these enzymes is composed of similar subunits. Examples of other enzymes composed of dissimilar subunits are ribulose diphosphate carboxylase (Rutner and Lane, 1967), tryptophan synthetase (Creighton and Yanofsky, 1966), and acetyl coenzyme A carboxylase (Alberts and Vagelos, 1968).

A simplified interpretation of the kinetic data and P_i:H_2 ratios observed in the ATP-dependent H_2 evolution reaction is presented in Figure 6. Steps 1–4 represent kinetically discernable events. E is intended to represent the reactive species of the enzyme following complex formation and no final stoichiometric composition of the components I and II is intended (even though in the computer treatment only two components were used). Only rate-controlling equilibria would be observed in the data. $E(ATP)_2$ is a hypothetical intermediate produced by the interaction or reaction of E with ATP preceding the branching steps 3 and 4. K_1, K_2, k_3, and k_4 correspond to equilibrium and rate constants associated with steps 1–4, respectively.

Step 1 is suggested by the sigmoidal nitrogenase rate profiles and the concept of dissimilar subunit interactions, E being in dynamic equilibrium with these components. In step 2, E binds two molecules of ATP to generate the intermediate $E(ATP)_2$. This is suggested by values for the interaction coefficients (n of eq 1) approaching 2. This interpretation is in line with the discussion of the physical significance of n by Atkinson *et al.* (1965) and is further supported by the previously cited observations of Moustafa and Mortenson (1967) with the acetylene reduction reaction, though they did not examine ATP levels below 2 mM.

Branching at $E(ATP)_2$ (steps 3 and 4) would reasonably explain the ATP:$2e^-$ values of 5 at 30° (Bulen and LeComte, 1966), its temperature dependence (Hadfield and Bulen, 1969), and its independence of ATP concentration (Table I). Steps 3 and 4 are competitive, both leading to P_i formation but only step 4 producing H_2 evolution, leaving an oxidized form of the enzyme, E′. It would be a rare coincidence if these pathways had identical energies of activation thus obviating the requirement that ATP:$2e^-$ values be integer numbers.

Since reductant is present in excess at all times during the reactions examined, the proposal is not intended to distinguish between reduction before or after association of I and II and indicates only the reduction following the loss of electrons in H_2 evolution. Each of the steps in this sequence may well consist of a series of intramolecular events. For example, H_2 evolution may proceed from $E(ATP)_2$ *via* a highly reactive intermediate, *e.g.*, a transition metal hydride subject to protonation. Such an occurrence would, however, not alter this introductory kinetic treatment. E′ is considered to be rapidly reduced to E by the excess $Na_2S_2O_4$ and ATP rapidly regenerated with the creatine kinase reaction thus maintaining a constant ATP concentration and essentially eliminating any ADP inhibition.

The relative rates of steps 3 and 4 determine the P_i:H_2 ratio. The corresponding rate equation becomes

$$\frac{\Delta P_i}{\Delta H_2} = \frac{2k_3[E(ATP)_2] + 2k_4[E(ATP)_2]}{k_4[E(ATP)_2]} = \frac{2k_3 + 2k_4}{k_4} \quad (2)$$

Equation 2 requires that the P_i:H_2 ratio be independent of ATP concentration, in agreement with the experimental data in Table I. This equation is also consistent with the temperature dependence of the P_i:H_2 ratio. A k_3/k_4 value at 30° of 1.5 yields the experimentally observed P_i:H_2 value of 5 at that temperature.

The Arrhenius equation, $k = Ae^{E/RT}$, may be substituted into eq 2 to yield eq 3 which, on algebraic and logarithmic treatment, leads to eq 4. The plot of log $(\Delta P_i/\Delta H_2) - 2$ *vs.*

$$\frac{\Delta P_i}{\Delta H_2} = \frac{2A_3e^{-E_3/RT} + 2A_4e^{-E_4/RT}}{A_4e^{-E_4/RT}} \quad (3)$$

$$\log\left(\frac{\Delta P_i}{\Delta H_2} - 2\right) = \log\frac{2A_3}{A_4} + \frac{E_4 - E_3}{2.303RT} \quad (4)$$

$1/T$ for the temperature data of Hadfield and Bulen (1969) gave within experimental error, the required straight line (Figure 7) from whose slope and intercept the *difference* in enthalpies of activation ($\Delta H_4^{\ddagger} - \Delta H_3^{\ddagger}$) and entropies of activation ($\Delta S_4^{\ddagger} - \Delta S_3^{\ddagger}$) between steps 3 and 4 were calculated to be −4.6 kcal mole^{-1} and −15.9 cal deg^{-1}, respectively. The less favorable $\Delta S^{\ddagger}$ for step 4 suggests the requirement for a more ordered structure in a transition state preceding H_2 formation.

TABLE III: Comparison of Experimental and Calculated Rates of H_2 Evolution.

Reaction	Nitrogenase[a]	ATP (mM)	mμmoles of H_2/ml per min v_{exp}[b]	v_{calcd}[c]
1	2A	0.025	3.1	2.6
2	2A	0.050	8.8	9.3
3	2A	0.075	18.6	18.2
4	2A	0.100	30.9	27.6
5	2A	0.125	36.7	36.5
6	4A	0.025	8.9	8.4
7	4A	0.050	35.9	28.6
8	4A	0.075	52.5	52.7
9	4A	0.100	82.0	76.0
10	4A	0.125	94.5	96.7
11	A	0.075	5.5	5.7
12	2A	0.075	18.6	18.2
13	4A	0.075	52.5	52.7
14	6A	0.075	84.7	92.4
15	A	0.125	12.3	12.6
16	2A	0.125	36.7	36.5
17	4A	0.125	94.5	96.7
18	6A	0.125	165.0	163.1
19	A	0.250	29.9	27.3
20	2A	0.250	64.5	68.5
21	4A	0.250	133.0	161.3
22	6A	0.250	185.1	260.7
23	0.25B	1.00	5.5	8.5
24	0.5B	1.00	16.8	19.5
25	1.0B	1.00	43.0	43.1
26	1.5B	1.00	69.7	67.8
27	2.0B	1.00	105	92.6
28	4.0B	1.00	198	195
29	6.0B	1.00	296	299
30	B	0.075	6.8	3.9
31	2B	0.075	18.1	13.1
32	4B	0.075	46.9	39.1
33	6B	0.075	78.4	70.5
34	8B	0.075	100	104
35	12B	0.075	163	174

[a] Nitrogenase concentration presented as A, 2A, 4A, etc. as described for Figures 1–4. For REMECH calculations, molar concentrations were based on an estimated minimal molecular weight of 160,000 calculated from Mo analysis. [b] Initial rates were based on 30- to 45-min reactions except when H_2 evolution exceeded manometer capacity and 4-ml reactions were substituted for 2-ml reactions with the appropriate corrections when low levels of enzyme or ATP made it advantageous. [c] Calculated as described in the text. The rate constant k_4 (turnover number at saturating ATP) used was 40 and 43 for series A and B, respectively.

Application of the computer program, REMECH, written for the general testing of reaction mechanisms (DeTar and DeTar, 1966, 1968) permits the calculation of species concentration (reactants, intermediates, and products) as a function of time when related to a proposed mechanism, equilibrium and rate constants, and initial reactant concentrations. With the reactions proposed in Figure 6 and assuming that E(ATP) is present in negligible concentration, for steps 1 and 2

$$K_1 = \frac{[E]}{[I][II]} \text{ and } K_2 = \frac{[E(ATP)_2]}{[E][ATP]^2} \quad (5)$$

For steps 3 and 4, k_4 was taken as the turnover number for H_2 evolution determined from the rates of H_2 evolution at saturating ATP and k_3 was taken as $1.5 \times k_4$ from the k_3/k_4 value of 1.5 at 30° based on the observed P_i:H_2 value of 5 previously discussed.

A systematic variation of K_1 and K_2 values gave $K_1 = 1 \times 10^4$ l. mole^{-1} and $K_2 = 3.1 \times 10^8$ (l. mole^{-1})^2 as the best fit of calculated rate data to experimental data (Table III).

Substitution of the calculated rate data (V calcd in Table III) for reactions 1–5 and 6–10 into the Hill equation gave n values of 1.85 and 1.80, respectively. The corresponding empirical values were 1.78 and 1.83 (Figure 3).

The question of the number of binding sites for ATP is clearly of interest. While the kinetic data are interpreted here in terms of an $E(ATP)_2$ complex, it is realized that rate profile data alone do not unequivocally establish the existence of multiple binding sites (Sweeny and Fisher, 1968). The present treatment differs from that for other allosteric enzymes in that all product formation was treated mechanistically as proceeding through $E(ATP)_2$ with precursor E(ATP) being considered simply as a transient intermediate.

A recently proposed common allosteric mechanism for active transport, muscle contraction, and ribosomal translocation by Hill (1969) in which the effector ligand is also the substrate, involves a conformational change induced by ATP, which is subsequently hydrolyzed to allow a steady-state repetition of the process. Perhaps in the case of nitrogenase this conformational change alters the ligand configuration about a transition metal creating the "active" species necessary for substrate reduction. Although the details of the mechanism here and those of parallel cases are not known, this type of function of ATP would seem to be worthy of additional consideration.

An extension of the suggested pathway leading to H_2 formation to one leading to the reduction of N_2 to NH_3 must, at this time, be made with caution even though (1) additional ATP is needed and (2) the rate of its hydrolysis is essentially constant irrespective of the reduction reaction being performed. Most striking, however, is the dependence of the product distribution, NH_3:H_2 ratio, upon the ATP concentration (Figure 4). At low levels of ATP, H_2O (or H^+) is reduced preferentially while at higher ATP levels NH_3 formation exceeds H_2 evolution even though the ATP:$2e^-$ ratio remains constant. One possible interpretation is that an ATP-dependent step (such as that depicted in Figure 6) occurs repeatedly and at a constant rate, providing different states of the enzyme (E_1, E_2, E_3) which are capable of reaction with different substrates involving the transfer of 2, 4, and 6 electrons, respectively.[3] Thus E_1 is equivalent to $E(ATP)_2$ of Figure 6, or the resulting reduced site capable of H_2 formation, while NH_3 formation is considered as proceeding *via* E_3 only. E_2 would then represent the

[3] Reaction in this case refers to reduced product formation and is not meant to preclude the binding of N_2 or other substrates to E.

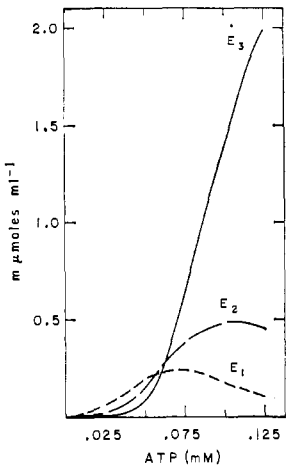

FIGURE 8: Steady-state concentrations of enzyme species (E_1, E_2, and E_3) after repeated reactions with ATP at varying ATP levels.

species capable of a four-electron reduction; its presence being related, *e.g.*, to the formation of CH_3NH_2 during cyanide reduction (Hardy and Knight, 1967). If each successive reaction with ATP produces reaction states which disappear upon substrate reduction, the steady-state concentration of these states should exhibit ATP dependence and E_3 should increase as E_1 and E_2 decrease. To check this interpretation of events, the following equilibria were analyzed with REMECH:

$$I + II \rightleftharpoons E \quad K = 1 \times 10^4 \text{ (l. mole}^{-1}\text{)}$$

$$E + 2ATP \rightleftharpoons E_1 \quad K = 3.1 \times 10^8 \text{ (l. mole}^{-1}\text{)}^2$$

$$E_1 + 2ATP \rightleftharpoons E_2 \quad K = 3.1 \times 10^8 \text{ (l. mole}^{-1}\text{)}^2$$

$$E_2 + 2ATP \rightleftharpoons E_3 \quad K = 3.1 \times 10^8 \text{ (l. mole}^{-1}\text{)}^2$$

Note that the K value for component association to form E is that determined from H_2 evolution data and that the K values for subsequent reactions with ATP are considered equivalent to that determined for the initial reactions providing the species from which H_2 is evolved.

Figure 8 shows the calculated steady-state concentrations of E_1, E_2, and E_3 when ATP concentrations were varied from 0.025 to 0.125 mM. As the ATP level is increased, E_1 and E_2 diminish and E_3 becomes the predominant species. The parallelism between the calculated steady-state concentrations of E_1, E_2, and E_3 and the experimentally observed formation of H_2 and NH_3 (Figure 4) lies in the shape of the curves (sigmoidal for E_3 and NH_3 formation, hyperbolic for E_1 and H_2 formation) and the crossover points which occur in the same general region of ATP concentrations.

Acknowledgments

The contributions of Dr. D. F. DeTar in the application of REMECH are greatly appreciated. The very able technical assistance of Mrs. Carolina Libby and Deloria Jacobs is gratefully acknowledged. We are grateful to J. R. LeComte for the nitrogenase complex preparations and to Robert Land for growing bacteria.

References

Alberts, A. W., and Vagelos, P. R. (1968), *Proc. Nat. Acad. Sci. U. S. 59*, 561.

Atkinson, D. E., Hathaway, J. A., and Smith, E. C. (1965), *J. Biol. Chem. 240*, 2682.

Bulen, W. A., Burns, R. C., and LeComte, J. R. (1964), *Biochem. Biophys. Res. Commun. 17*, 265.

Bulen, W. A., Burns, R. C., and LeComte, J. R. (1965), *Proc. Nat. Acad. Sci. U. S. 53*, 532.

Bulen, W. A., and LeComte, J. R. (1966), *Proc. Nat. Acad. Sci. U. S. 56*, 979.

Bulen, W. A., and LeComte, J. R. (1970), *Methods Enzymol.* (in press).

Burns, R. C., and Bulen, W. A. (1965), *Biochim. Biophys. Acta 105*, 437.

Creighton, T. E., and Yanofsky, C. (1966), *J. Biol. Chem. 241*, 980.

DeTar, D. F., and DeTar, C. E. (1966), *J. Phys. Chem. 70*, 3842.

DeTar, D. F., and DeTar, C. E. (1968), *in* Computer Programs in Chemistry, Vol 2, DeTar, D. F., Ed., New York, N. Y., W. A. Benjamin.

D'Eustachio, A. J., and Hardy, R. W. F. (1964), *Biochem. Biophys. Res. Commun. 15*, 319.

Fisher, H. F., and Bard, J. R. (1970), *Biochim. Biophys. Acta* (in press).

Furchgott, R. F., and de Gubareff, T. (1956), *J. Biol. Chem. 223*, 377.

Good, N. E., Winget, G. D., Winter, W., Connolly, T. N., Izawa, S., and Singh, R. M. M. (1966), *Biochemistry 5*, 467.

Gornall, A. G., Bardawill, C. J., and David, M. M. (1949), *J. Biol. Chem. 177*, 751.

Grisolia, S., Fernandez, M., Amelunxen, R., and Quijada, C. L. (1962), *Biochem. J. 85*, 568.

Hadfield, K. L., and Bulen, W. A. (1969), *Biochemistry, 8*, 5103.

Hardy, R. W. F., Holster, R. D., Jackson, G. K., and Burns, R. C. (1968), *Plant Physiol. 43*, 1185.

Hardy, R. W. F., and Knight, E. (1967), *Biochim. Biophys. Acta 139*, 69.

Hill, T. L. (1969), *Proc. Nat. Acad. Sci. U. S. 64*, 267.

Huang, C. Y., and Graves, D. J. (1970), *Biochemistry 9*, 660.

Monod, J., Changeux, J.-P., and Jacob, F. (1963), *J. Mol. Biol. 6*, 306.

Mortenson, L. E. (1964), *Proc. Nat. Acad. Sci. U. S. 52*, 272.

Mortenson, L. E., Morris, J. A., and Jeng, P. Y. (1967), *Biochim. Biophys. Acta 141*, 516.

Moustafa, E., and Mortenson, L. E. (1967), *Nature (London) 216*, 1241.

Nihei, T., Noda, L., and Morales, M. F. (1961), *J. Biol. Chem. 236*, 3203.

Rutner, A. C., and Lane, M. D. (1967), *Biochem. Biophys. Res. Commun. 28*, 531.

Sweeney, J. R., and Fisher, J. R. (1968), *Biochemistry 7*, 561.