of mammalian cells. The active factor appears to be a protein that interferes with the poly(A)-protein interaction in the complex. We provide evidence that this factor renders the poly(A) susceptible to snake venom exonuclease. The hydrolytic agent in the cytoplasm may be an exonuclease as well, but further work with more purified preparations will be required to clarify its mode of action. The inhibitory effect of exogenous RNA on poly(A) hydrolysis was observed with the cytoplasmic preparation as well as with the snake venom enzyme.

Our results indicate the possibility of a control mechanism based on differential susceptibility of the poly(A) segment to degradation in the cytoplasm. Some suggestion of differential poly(A) stability is provided by the data on the time course of hydrolysis in Figure 2. More precise data will be required, preferably with unique mRNA species, in order to verify this possibility.

Acknowledgments

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References

Blobel, G. (1973), *Proc. Natl. Acad. Sci. U.S.A. 70,* 924.
Brawerman, G. (1973a), *Mol. Biol. Rep. 1,* 7.
Brawerman, G. (1973b), International Symposium on Normal and Pathological Protein Synthesis in Higher Organisms, INSERM, Paris.
Brawerman, G., and Diez, J. (1975), *Cell 5,* 271.
Brawerman, G., Mendecki, J., and Lee, S. Y. (1972), *Biochemistry 11,* 637.
Greenberg, J. R., and Perry, R. P. (1972), *Biochim. Biophys. Acta 287,* 361.
Jeffery, W. R., and Brawerman, G. (1974), *Biochemistry 13,* 4663.
Kwan, S. W., and Brawerman, G. (1972), *Proc. Natl. Acad. Sci. U.S.A. 69,* 3247.
Lee, S. Y., Krsmanovic, V., and Brawerman, G. (1971a), *Biochemistry 10,* 895.
Lee, S. Y., Mendecki, J., and Brawerman, G. (1971b), *Proc. Natl. Acad. Sci. U.S.A. 68,* 1331.
Mendecki, J., Lee, S. Y., and Brawerman, G. (1972), *Biochemistry 11,* 792.
Penman, S., Vesco, C., and Penman, M. (1968), *J. Mol. Biol. 34,* 49.
Perry, R. P., La Torre, J., Kelley, D. E., and Greenberg, J. R. (1972), *Biochim. Biophys. Acta 262,* 220.
Sheiness, D., and Darnell, J. E. (1973), *Nature (London), New Biol. 241,* 265.
Soreq, H., Nudel, U., Salomon, R., Revel, M., and Littauer, U. Z. (1974), *J. Mol. Biol. 88,* 233.

Kinetics of Dithionite Ion Utilization and ATP Hydrolysis for Reactions Catalyzed by the Nitrogenase Complex from *Azotobacter vinelandii*†

Gerald D. Watt* and Andrea Burns

ABSTRACT: The kinetics of $S_2O_4^{2-}$ utilization and ATP hydrolysis during the nitrogenase-catalyzed H_2 evolution and acetylene and nitrogen-reducing reactions were studied using a polarographic technique to monitor $S_2O_4^{2-}$ concentration. Rate constants for both $S_2O_4^{2-}$ utilization and ATP hydrolysis were determined as a function of temperature and corresponding activation energies determined. The activation energy for ATP hydrolysis differs from that for product formation or $S_2O_4^{2-}$ utilization by 5 kcal/mol above 20 °C and by 25 kcal/mol below 20 °C. The rate law for $S_2O_4^{2-}$ utilization was determined and describes the enzyme catalyzed rate over a 1000-fold variation in $S_2O_4^{2-}$ concentration and at least a 100-fold change in ATP concentration. The rate law for $S_2O_4^{2-}$ uilization under N_2-reducing conditions at 25 °C is given by $-d([S_2O_4^{2-}])/dt = (2.3 \times 10^{-3}E_T[S_2O_4^{2-}]^{1/2}[ATP]^2)/([ATP]^2 + K_1[ATP] + K_2)$, where E_T is total enzyme concentration in mg/ml and K_1 and K_2 are equilibrium constants for ATP binding to nitrogenase. The half-order dependence of the rate on $S_2O_4^{2-}$ concentration is interpreted in terms of the equilibrium $S_2O_4^{2-} = 2SO_2^-$, in which SO_2^- is the actual electron donor to nitrogenase. A partial mechanism incorporating these results is presented.

Nitrogenase is capable of reducing a wide range of substrates possessing unique and diverse properties. These include neutral gaseous molecules (N_2, N_2O, and acetylene), relatively large organic molecules (isonitriles, monosubstituted acetylenes, and nitriles), and anions (CN^-, N_3^-). In the absence of any of the above, $H_2O(H^+)$ serves as a substrate. In addition to these reducible substrates, nitrogenase also requires: (1) a source of low potential electrons, with $E°$ more negative than −400 mV (Watt and Bulen, 1974; Evans and Albrecht, 1974); and (2) $MgATP^{2-}$ which is hydrolyzed to $MgADP^{2-}$ and P_i.[1]

Zumft and Mortenson (1975) recently reviewed the properties of nitrogenases from bacterial sources and discussed the meager kinetic results so far reported and the problems associated with studying this aspect of nitrogenase catalysis. The rate dependence on ATP for nitrogenase-catalyzed reactions

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[1] Abbreviations used: P_i, inorganic phosphate produced from ATP hydrolysis; ATP, adenosine 5′-triphosphate; Tes, *N*-tris(hydroxymethyl)-methyl-2-aminoethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; EPR, electron paramagnetic resonance.

is unclear, particularly at low ATP concentrations where both sigmoidal (Moustafa and Mortenson, 1967; Silverstein and Bulen, 1970; Bergerson and Turner, 1973) and nonsigmoidal (Thorneley and Willison, 1974; Ljones, 1973) kinetics have been reported. Even less kinetic information is available concerning the rate at which $S_2O_4^{2-}$ or other electron donors are used by nitrogenase.

The rate at which reduced products are formed by nitrogenase has been reported (Burns, 1969; Silverstein and Bulen, 1970; Shilov and Likhtenshtein, 1971; Ljones, 1973) to be independent of the nature of the substrate for N_2 reduction or H_2 evolution. Ljones (1973) reported that CN^- and N_3^- inhibited the rate of utilization of $S_2O_4^{2-}$ but attributed this inhibition to protein destruction by these substrates. These results and others (Zumft and Mortenson, 1975) suggest that substrates are reduced at a constant rate by nitrogenase. The consequence of this is that substrate reduction is not likely to be involved in the rate-limiting step of nitrogenase catalysis. The answer to the question, "what is the rate-limiting step in nitrogenase catalysis", is thus not clear and becomes important in attempting to understand the mechanism of nitrogenase action. The detailed form of the rate law describing the utilization (production) of the various nitrogenase substrates (products) is important in any discussion of the rate-limiting step of enzyme catalysis as well as in attempts to explain the mechanism of enzyme action.

We have previously reported (Watt et al., 1975) that polarographic and calorimetric techniques provide versatile and sensitive methods for detecting nitrogenase activity. These methods have now been developed (Watt, 1976) for the study of the kinetics of nitrogenase activity over a wide range of substrate concentrations and for the determination of the rate laws governing their utilization. This paper describes the results of these kinetic investigations.

Experimental Section

Materials. Adenosine 5′-triphosphate (ATP) and (hydroxymethyl)methyl-2-aminoethanesulfonic acid (Tes) were purchased from the Sigma Chemical Co. Creatine kinase was purchased from Nutritional Biochemical Corp. Creatine phosphate was synthesized in this laboratory by Dr. James Corbin. Sodium dithionite, $Na_2S_2O_4$ (98%), was purchased as a special order from Associated Chemical Co. Ltd., Harrogate, Yorkshire, England. The sealed metal container was opened and subsequently stored in a Vacuum Atmospheres Corp. drybox under oxygen-free argon. Smaller samples of $Na_2S_2O_4$ for laboratory use were removed from the stock supply as needed and stored in argon-filled desiccators over anhydrous $CaCl_2$. When stored with these precautions, the $Na_2S_2O_4$ remained >95% pure for periods greater than 2 years as measured by amperometric or calorimetric titrations against $K_3Fe(CN)_6$ or riboflavin 5′-phosphate. Argon and prepurified nitrogen were passed through a hot quartz tube (600 °C) containing copper turnings to remove oxygen prior to use. Preparations of nitrogenase *complex* from *A. vinelandii* were obtained as previously described (Bulen and LeComte, 1966, 1972, unpublished results) and possessed activities of 200–300 nmol of H_2 evolved per min per mg of complex. ATP/2e$^-$ values of 4–5 at 25 °C were usually obtained with these enzyme preparations.

Methods. All solutions were prepared anaerobically using airless glassware and transferred with Hamilton gas-tight syringes. N_2 reduction was measured as described previously (Bulen and LeComte, 1966). H_2 evolution was measured either manometrically or with a Hewlett-Packard Model 5750 research gas chromatograph (molecular sieve 5A, argon carrier gas). Ethylene formation was determined by gas chromatography (alumina or Poropak-N).

A creatine phosphate–creatine kinase ATP generating system was used to maintain a constant ATP concentration and to prevent ADP from reaching inhibitory levels. No variation in the rate of $S_2O_4^{2-}$ utilization at any of the temperatures studied was observed upon increasing the creatine kinase levels by a factor of 4. The P_i released was measured as previously described (Bulen and LeComte, 1966) using controls minus $Na_2S_2O_4$.

The rates of $S_2O_4^{2-}$ utilization by the nitrogenase catalyzed H_2-evolution, acetylene-reduction, and N_2-reduction reactions were measured polarographically. The polarographic apparatus and procedure for measuring $S_2O_4^{2-}$ concentration have been described (Watt et al., 1975). A standard assay solution of 5.0 ml was used which consisted of Tes or Tris buffer (250 μmol), pH 8, creatine phosphate (260 μmol), creatine kinase (5 nmol), and variable amounts of both $S_2O_4^{2-}$ and ATP. The Mg^{2+} concentration always exceeded 3.0 mM and had no effect on the rate of $S_2O_4^{2-}$ utilization in the range of 1.0 to 5.0 mM at a given ATP concentration. Under the above experimental conditions, the $MgATP^{2-}$ complex was the only important ATP-containing species present during the reactions (see Thorneley and Willison, 1974).

The rate of $S_2O_4^{2-}$ oxidation in this standard assay solution was measured: (1) at various ATP concentrations maintained constant by the ATP-generating system; (2) at various temperatures; and (3) under various atmospheres (N_2, H_2, Ar, CO, or acetylene) by adding 0.2 ml of nitrogenase enzyme (15–30 mg/ml) to the polarographic cell which contained 5.0 ml of the equilibrated, oxygen-free assay solution. The kinetics of $S_2O_4^{2-}$ utilization was followed by continuously recording the anodic diffusion current of $S_2O_4^{2-}$ until all $S_2O_4^{2-}$ was consumed (15–25 min at temperatures ≥25 °C and 25–45 min at temperatures below 25 °C). Some experiments using a Durrum stopped-flow spectrophotometer followed the oxidation of $S_2O_4^{2-}$ optically at 320 nm.

The rate of H_2 evolution with limiting $S_2O_4^{2-}$ was measured manometrically (Bulen and LeComte, 1966). The concentration of $S_2O_4^{2-}$ in the standard assay solution was determined polarographically and 5.0 ml of this solution was transferred by gas-tight syringe to the manometers, previously degassed and containing enzyme. When equilibrated, the enzyme and assay solution were mixed and readings were taken every minute as the enzyme-catalyzed reaction consumed $S_2O_4^{2-}$ and produced H_2.

Results

Figure 1 shows a polarographic trace of $S_2O_4^{2-}$ utilization during the hydrogen-evolution reaction catalyzed by nitrogenase. The anodic current before enzyme is added (arrow) gives the initial $S_2O_4^{2-}$ concentration in the assay mixture. The constancy of this current with time demonstrates that no $S_2O_4^{2-}$-consuming reactions are occurring in the absence of enzyme catalysis. After enzyme is added an induction period was noted (not shown in Figure 1) in many of the reactions during which no immediate $S_2O_4^{2-}$ utilization occurred even though all of the reactants were present for the enzyme-catalyzed reactions to proceed. The time interval of this induction period is very temperature sensitive, becoming shorter at temperatures above 20 °C and longer below this temperature. The presence of this induction period (with those enzyme preparations which possessed it) depends on the sequence in which the enzyme and reagents of the assay solution are mixed.

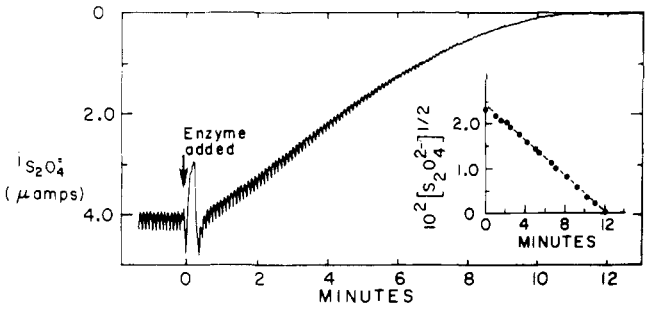


FIGURE 1: A polarographic trace of the nitrogenase-catalyzed utilization of $S_2O_4^{2-}$ during the H_2-evolution reaction. The ordinate is the anodic diffusion current of $S_2O_4^{2-}$ and is directly proportional to $S_2O_4^{2-}$ concentration ($[S_2O_4^{2-}] = 1.375 \times 10^{-4} i_{S_2O_4^{2-}}$). The reaction is initiated by adding enzyme to the cell (arrow) containing all reactants necessary for the nitrogenase-catalyzed, H_2-evolution reaction (see text for details). The insert is a plot of $[S_2O_4^{2-}]^{1/2}$ against time in minutes.

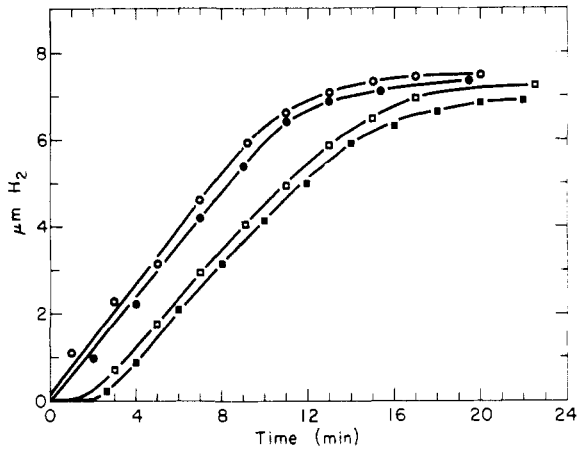


FIGURE 2: The nitrogenase catalyzed H_2-evolution reaction at 25 °C under the conditions of limiting $[S_2O_4^{2-}]$. The upper set of curves (○, ●) was obtained by adding 0.20 ml of nitrogenase complex preincubated (10 min) with $MgATP^{2-}$ to manometers containing the ATP-generating system and $[S_2O_4^{2-}]$ initially at 5.0×10^{-3} M. The lower set of curves (□, ■) was obtained as above, except preincubation of the nitrogenase complex was omitted. The ratio of the total H_2 measured to total $S_2O_4^{2-}$ oxidized in the four kinetic experiments shown varied from 0.96 to 1.08.

It occurs if the enzyme is added last to the assay mixture, as was done for most of the results reported here.

If enzyme and $MgATP^{2-}$ are preincubated before addition to the assay solution in the polarographic cell, the induction period is eliminated and $S_2O_4^{2-}$ utilization begins immediately. Preincubation of enzyme with other constituents of the assay solution were ineffective in eliminating the induction period.

Although adding enzyme last to the assay mixture introduces some uncertainty in analyzing the first minute or so of the enzyme-catalyzed reaction, the remaining 15 to 30 min are more than adequate to properly evaluate the rate of reaction, and the overall convenience and reproducibility gained by this procedure makes the omission of this initial part of the curve justifiable.

Following the induction period (when present) $S_2O_4^{2-}$ utilization begins and the anodic current ($S_2O_4^{2-}$ concentration) decreases with time as shown in Figure 1. Treatment by usual kinetic procedures indicates that this curve corresponds to a one-half order reaction with respect to $S_2O_4^{2-}$ concentration. This dependence is strictly obeyed to the conclusion of the reaction (see insert to Figure 1). Curves identical with

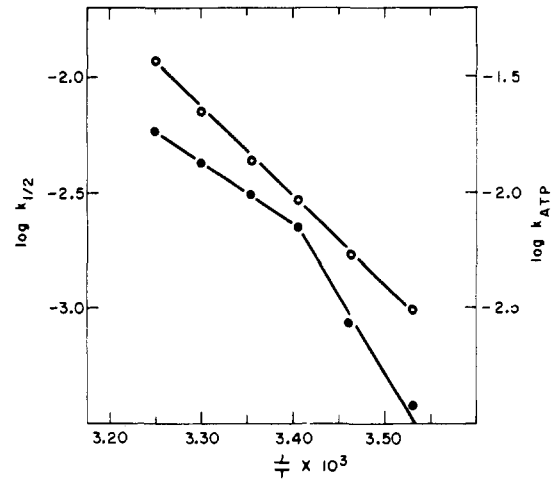


FIGURE 3: Arrhenius plots for the one-half order rate constants ($k_{1/2}$) for $S_2O_4^{2-}$ utilization (●) and the rate constants (k_{ATP}) for ATP hydrolysis (○). The ATP concentration is 5×10^{-3} M at all temperatures and is maintained constant by the ATP-generating system. E_a for ATP hydrolysis (○) was found to be 17.2 kcal/mol, while values of 12.3 and 35 kcal/mol were found for the biphasic $S_2O_4^{2-}$ utilization curve (●).

those of Figure 1 were obtained when the enzyme-catalyzed utilization of $S_2O_4^{2-}$ in identical reaction mixtures was run under N_2, Ar, H_2, CO, or acetylene atmospheres.

Figure 2 shows that similar kinetic behavior is seen by measuring the rate at which H_2 is evolved by the enzyme-catalyzed, H_2-evolution reaction. Preincubation of enzyme and $MgATP^{2-}$ causes H_2 evolution to begin immediately, whereas an induction period is seen in the absence of preincubation. Once H_2 evolution begins, its rate occurs uniformly at first but then decreases with time as $S_2O_4^{2-}$ is consumed. The rate of H_2 evolution for the last part of the curve in Figure 2 appears to be a function of $[S_2O_4^{2-}]^{1/2}$, i.e., $d[H_2]/dt = k[S_2O_4^{2-}]^{1/2}$, whereas the rate of H_2 evolution for the initial part of the curve is independent of $[S_2O_4^{2-}]$ (zero order in $[S_2O_4^{2-}]$). It is clear from Figure 2 that 1 mol of H_2 is evolved per mol of $S_2O_4^{2-}$ consumed.

Variation of Rate with Temperature. The one-half order rate constants at various temperatures for $S_2O_4^{2-}$ utilization by the nitrogenase-catalyzed, H_2-evolution reaction, maintained at 5 mM ATP by the ATP generating system, are given in Figure 3 in the form of an Arrhenius plot. The rate constants for phosphate (P_i) production resulting from ATP hydrolysis at the same temperatures are also included in this figure. The rate constants for ATP hydrolysis at the various temperatures studied were calculated from eq 1

$$\frac{d[P_i]}{dt} = -\left(\frac{[ATP]}{[S_2O_4^{2-}]}\right)\left(\frac{d[S_2O_4^{2-}]}{dt}\right) \qquad (1)$$

which relates the rates of $S_2O_4^{2-}$ utilization, measured in this study, to the ATP/$S_2O_4^{2-}$ value determined previously as a function of temperature (Watt et al., 1975). The one-half order rate constants for $S_2O_4^{2-}$ utilization produce a biphasic plot, while the rate constants for P_i formation form a single straight line.

Activation energies of +35 and +12.3 kcal/mol for $S_2O_4^{2-}$ utilization are obtained from the 10–20 °C and the 20–35 °C temperature regions, respectively. These results are in agreement with those previously reported for *Azotobacter vinelandii* nitrogenase (Burns, 1969; Shilov and Likhtenshtein, 1971) obtained from the rates of product formation.

The Arrhenius plot for the rate of P_i production (ATP hy-

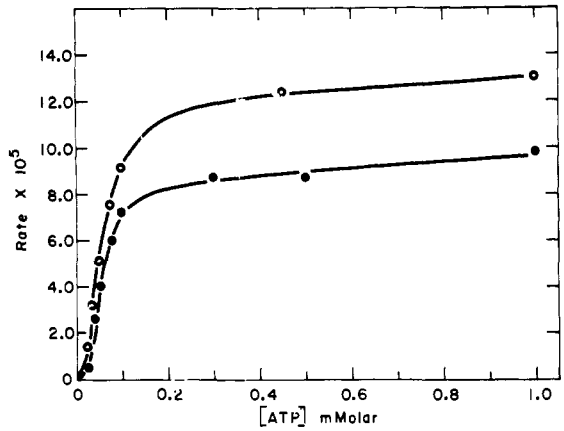


FIGURE 4: The rate of the nitrogenase-catalyzed, H_2-evolution reaction at 25 (●) and 30 °C (○) as a function of ATP concentration in the presence of the ATP-generating system and 10-20 mM initial $S_2O_4^{2-}$ concentrations. The ordinate is the rate (molar $S_2O_4^{2-}$/min) at which $S_2O_4^{2-}$ is consumed by the nitrogenase-catalyzed reactions as measured polarographically.

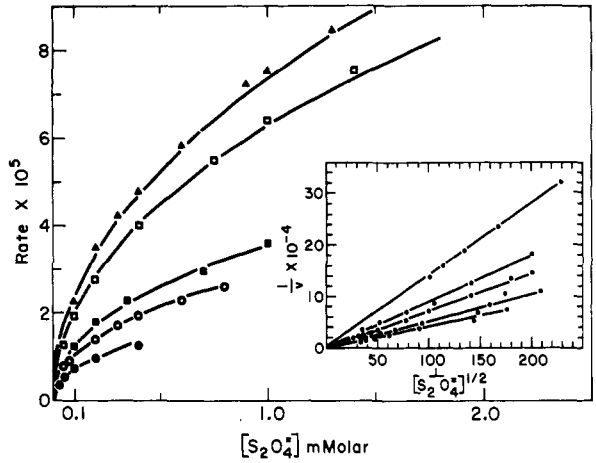


FIGURE 5: The rate of $S_2O_4^{2-}$ utilization (molar $S_2O_4^{2-}$/min) at 25 °C under N_2-reducing conditions (1 atm N_2) as a function of both $S_2O_4^{2-}$ and ATP concentrations. The solid line is calculated from eq 2 and the data points are obtained experimentally. The insert is a double-reciprocal plot of 1/rate vs. $1/[S_2O_4^{2-}]^{1/2}$. The ATP concentrations beginning with the lower curve and increasing are: 5×10^{-5}, 7.5×10^{-5}, 1.3×10^{-4}, 5×10^{-4}, and 1×10^{-3} M.

drolysis) catalyzed by nitrogenase yields an activation energy of +17.2 kcal/mol. This result taken together with those for $S_2O_4^{2-}$ utilization discussed above clearly indicate that the rates of ATP hydrolysis and electron transfer from $S_2O_4^{2-}$ to products catalyzed by nitrogenase are affected differently by variations in temperature. In fact, at 10 °C the rate of $S_2O_4^{2-}$ utilization is barely detectable, while ATP hydrolysis occurs at an easily measurable rate. *At this temperature or below, nitrogenase is essentially an ATPase incapable of transferring electrons to substrate.*

The rate of $S_2O_4^{2-}$ utilization at 25 °C for acetylene and nitrogen reduction produces curves identical with that for H_2 evolution shown in Figure 1. Identical rate constants with the same temperature variations (same activation parameters as those for H_2 evolution) were observed from acetylene and nitrogen reduction.

Rate Law for $S_2O_4^{2-}$ and ATP Utilization during H_2 Evolution. Figure 4 shows the relationship between the rate of $S_2O_4^{2-}$ utilization and ATP concentration for the H_2-evolution reaction. Sigmoidal curves are clearly evident at both 25 and 30 °C. Above 0.3 mM the rate becomes insensitive to increasing [ATP], indicating enzyme saturation with respect to ATP. Below 0.01 mM ATP, no $S_2O_4^{2-}$ utilization could be detected, even though all components necessary for nitrogenase activity were present. These same results were obtained under N_2-reducing conditions (1 atm N_2), where a more complete and thorough study was done (see later).

The data in Figure 4 at initial $S_2O_4^{2-}$ concentrations greater than 10 mM are fit empirically by eq 2

$$\frac{-d[S_2O_4^{2-}]}{dt} = \frac{E_T A[S_2O_4^{2-}]^{1/2}[ATP]^2}{[ATP]^2 + K_1[ATP] + K_2} \quad (2)$$

where K_1, K_2, and A at 25 °C are numerically equal to 5.05×10^{-5} M^{-1}, 2.8×10^{-9} M^{-1}, and 2.35×10^{-3} $M^{1/2}$ ml min^{-1} mg^{-1}, respectively. E_T is the concentration of nitrogenase complex expressed in mg/ml. An equation of this same form with $A = 3.1 \times 10^{-3}$ $M^{1/2}$ ml min^{-1} mg^{-1}, $K_1 = 2.0 \times 10^{-5}$ M^{-1}, and $K_2 = 1.3 \times 10^{-9}$ M^{-1} describes the rate of $S_2O_4^{2-}$ utilization at 30 °C.

Rate Law for $S_2O_4^{2-}$ and ATP Utilization during N_2 Reduction. The rate of $S_2O_4^{2-}$ utilization by nitrogenase as a function of $S_2O_4^{2-}$ concentration under N_2-reducing conditions (1 atm N_2) and at various ATP concentrations is shown in Figure 5. The solid lines are calculated from eq 2 and provide a good fit to the experimental points over a 100-fold variation in ATP and a 1000-fold variation in $S_2O_4^{2-}$ concentration. The close agreement between experimental and calculated results provides a basis for using eq 2 for computer-assisted analysis of our kinetic data.

Although eq 2 was derived under H_2 evolving conditions, it also accurately describes the rate of $S_2O_4^{2-}$ utilization for N_2 and acetylene reduction and indicates that the rate of enzyme catalysis is independent of the type or concentration of the reducible substrate. Therefore, the reducible substrate must either interact with the enzyme prior to or independently of the ATP and $S_2O_4^{2-}$ reactions. Once the enzyme is poised by the ATP-$S_2O_4^{2-}$ reactions, it is irreversibly committed to react and any reducible substrate which is available undergoes reduction.

The curves in Figure 5 are truly parabolic as is seen from the insert where the reciprocal rate is plotted against $1/[S_2O_4^{2-}]^{1/2}$. The half-order dependence of the rate on $[S_2O_4^{2-}]$ suggests that SO_2^- is the actual reducing agent, formed by eq 3 (Lambeth and Plamer, 1973; Creutz and Sutin, 1974).

$$S_2O_4^{2-} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} 2SO_2^- \quad (3)$$

$$K = 1.4 \times 10^{-9}\ M^{-1},$$

$$k_1 = 1.7\ s^{-1},$$

$$k_{-1} = 1.2 \times 10^9\ M^{-1}\ s^{-1}$$

The slopes of the lines in the insert are clearly functions of the ATP concentration. This function was evaluated and corresponds to eq 4.

$$\text{slope} = 425\,\frac{[ATP]^2 + K_1[ATP] + K_2}{[ATP]^2} \quad (4)$$

A replot of these slopes against the reciprocal of the ATP concentration gives a straight line from whose intercept a value of 2.35×10^{-3} $M^{1/2}$ ml min^{-1} mg^{-1} was obtained. This $K_{SO_2^-}/V_{SO_2^-}$ value is well determined but individual values of $K_{SO_2^-}$ and $V_{SO_2^-}$ could not be evaluated from our data because of the indeterminate condition (both $K_{SO_2^-}$ and $V_{SO_2^-}$

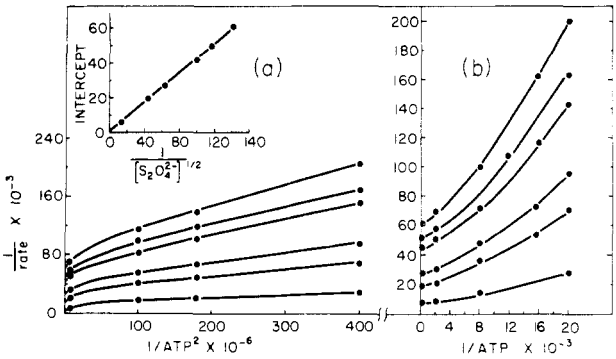


FIGURE 6: (a) A plot of 1/rate against 1/[ATP] for nitrogenase catalysis at 25 °C, pH 8.0 and 1 atm N_2. The solid lines are calculated from eq 2. The points (●) are representative experimental data. (b) Same as a, except 1/rate is plotted against $1/[ATP]^2$. The insert is a replot of the intercepts in either a or b plotted against $1/[S_2O_4^{2-}]^{1/2}$. The $S_2O_4^{2-}$ concentrations beginning with the lower curve and increasing are: 5×10^{-5}, 7.5×10^{-5}, 1×10^{-4}, 5×10^{-4}, 1×10^{-3}, and 5×10^{-3} M.

appear to be infinite) resulting from all lines in the insert to Figure 5 intersecting at zero. The intersection of all lines at zero indicates that nitrogenase never becomes saturated with $S_2O_4^{2-}$ (or SO_2^-) even at infinite $S_2O_4^{2-}$ concentrations. In other words, SO_2^- reacts "chemically" (without enzyme-reductant complex formation) with nitrogenase.

Plots of reciprocal rate vs. 1/[ATP] and $1/[ATP]^2$ are shown in Figures 6a and 6b, respectively. A replot of the intercepts of these figures vs. $1/[S_2O_4^{2-}]^{1/2}$ is shown as an insert in Figure 6a.

A straight line is obtained for both plots which intersects at the origin. The $K_{SO_2^-}/V_{SO_2^-}$ value is obtained from the slope of these plots, but separate values of $V_{SO_2^-}$ and $K_{SO_2^-}$ again appear to be infinite.

Discussion

Nitrogenase requires three substrates for activity: (1) a reducible small molecule (N_2, CN^-, acetylene, H_2O, etc.), (2) a source of low potential electrons, which in the present study is supplied by $S_2O_4^{2-}$, and (3) $MgATP^{2-}$ which becomes hydrolyzed to $MgADP^{2-}$ and P_i. The rate laws describing the utilization of each of these substrates and the formation of products are necessary information in eventually understanding the mechanism by which nitrogenase functions. Certain aspects of each of these substrates interacting with nitrogenase are discussed separately and then later discussed collectively in terms of the mechanism of nitrogenase action which they suggest.

Small Molecule Substrates. Previous kinetic studies of the N_2-reducing and H_2-evolving reactions catalyzed by *Azotobacter vinelandii* nitrogenase (Burns, 1969; Silverstein and Bulen, 1970) have shown that the rate of production of reducing equivalents (measured by the rate of product formation) are identical. Ljones (1973) reported that the rate of $S_2O_4^{2-}$ utilization catalyzed by *Clostridium pasteurianum* nitrogenase was constant whether under N_2-reducing or H_2-evolving conditions. We have confirmed the above results by measuring the rate of $S_2O_4^{2-}$ utilization polarographically (for the H_2-evolution reaction, the rate of product formation was also measured) and have shown further that acetylene reduction also occurs at this same rate. These three substrates [N_2, $H_2O(H^+)$, acetylene] are sufficiently different in their properties, reduction products, and requirements for electrons that identical rates of reduction would not be expected. Consequently, the rate of substrate reduction is not rate limiting as has been suggested by Zumft et al. (1974) but instead this distinction belongs to some other reaction which controls the rate.

$S_2O_4^{2-}$ Substrate. The kinetics of enzyme catalysis was measured by determining the rate of $S_2O_4^{2-}$ utilization in the presence of a constant ATP concentration maintained by the ATP-generating system. Under these conditions the dependence of the rate on ATP concentration should be zero order if the ATP concentration is maintained constant and the observed order of the reaction is that due to $S_2O_4^{2-}$. The ATP concentration remained constant throughout the reaction as evidenced by: (1) the strictly obeyed one-half-order reaction for the entire reaction interval (Figure 1) and (2) the lack of variation in rate as components of the ATP-generating system were increased by a factor of 4.

This one-half-order dependence on $[S_2O_4^{2-}]$ is important in understanding how low-potential electrons are used by nitrogenase. Lambeth and Palmer (1973) and Creutz and Sutin (1974) have discussed the different mechanisms by which $S_2O_4^{2-}$ acts as a reducing agent. Lambeth and Palmer (1973) concluded from a kinetic study of the reduction of a series of redox proteins by $S_2O_4^{2-}$ that the almost exclusively preferred mechanism involves SO_2^- radicals with rate constants in the range 10^7 M^{-1} s^{-1}. The dependence of the rate on $S_2O_4^{2-}$ for these redox reactions was reported to be one-half order. The recent kinetic study reported by Thorneley et al. (1976) indicates that SO_2^- reacts with dye-oxidized Fe protein from *Klebsiella pneumoniae* with a rate constant $>10^8$ M^{-1} s^{-1}. The observed one-half order dependence of $[S_2O_4^{2-}]$ utilization reported here for nitrogenase catalysis is therefore consistent with SO_2^- being the reductant for the Fe protein. Consistent with this view, we have found that the addition of Fe protein to reaction mixtures being catalyzed by the *Azotobacter* nitrogenase *complex* increases the rate of $S_2O_4^{2-}$ utilization, although at present the exact dependence of the rate on the Fe protein concentration has not been determined.

Whatever the electron accepter is on nitrogenase, we consider its acceptance of an electron from SO_2^- to be essentially irreversible for two reasons. The first is that the difference between the midpoint potential of $S_2O_4^{2-}$ and nitrogenase at pH 7 is on the order of 200 mV for an $n = 2$ process in favor of reduction. The second and probably the most important is that the product of $S_2O_4^{2-}$ oxidation is SO_3^{2-} which at neutral or alkaline pH is not easily reduced back to $S_2O_4^{2-}$ (Meites, 1955).

The rate of H_2 evolution in Figure 2 at low $S_2O_4^{2-}$ concentration becomes curved and can be shown to depend on the square root of the $S_2O_4^{2-}$ concentration. However, at high $S_2O_4^{2-}$ concentration the linear relationship in Figure 2 indicates that the rate of H_2 evolution is independent of $S_2O_4^{2-}$ concentration. These results are similar to those of Ljones and Burris (1972) who reported that the rates of $S_2O_4^{2-}$ utilization and H_2 evolution using *Clostridium pasteurianum* nitrogenase were independent of $S_2O_4^{2-}$ concentration. Only at very low $S_2O_4^{2-}$ concentrations is loss of linearity observed in their data for H_2 evolution which seems to correspond to a square root dependence of the rate on $S_2O_4^{2-}$ concentration. The result reported here and those reported by Ljones and Burris (1972), indicating that the rate of product formation is independent of $S_2O_4^{2-}$ concentration, suggests that the rate of product formation may be limited by the rate at which ATP and nitrogenase interact and not by $S_2O_4^{2-}$ interaction except when the latter becomes limiting.

$MgATP^{2-}$ Substrate. According to Thorneley and Willison (1974), $MgATP^{2-}$ is the form of ATP used by nitrogenase

while Mg_2ATP is inactive. Our experiments were designed such that $MgATP^{2-}$ was the predominant ATP-containing species thereby minimizing complicating effects on the observed rates. Our results show definitely that sigmoidal kinetics occur for the acetylene-reducing, N_2-reducing, and H_2-evolving reactions catalyzed by *Azotobacter vinelandii* nitrogenase. Silverstein and Bulen (1970) and Moustafa and Mortenson (1967) had previously reported this effect for nitrogenase from *Azotobacter vinelandii* and *Clostridium pasteuranum* from rates of H_2-evolution and N_2-reduction reactions but the results of the present study using more sensitive methods unequivocally demonstrate this behavior.

Studies using nitrogenases from *Clostridium pasteuranum* (Ljones, 1973) and *Klebsiella pneumoniae* (Thorneley and Willison, 1974) have indicated that nonsigmoidal kinetics were observed. These studies were not performed at ATP concentrations as low as those in this study nor with the sensitivity to detect reaction as was available to us. We suggest that these previous studies could have overlooked this effect using these other enzymes. For example, the Fe protein from *Clostridium pasteuranum* is known to bind two ATP molecules with dissociation constants of 17 μm (Tso and Burris, 1973). Using these data, the concentrations of free Fe protein and its ATP-containing forms can be calculated as a function of ATP concentration. These calculations suggest that sigmoidal behavior should be observable at 0.02 mM ATP or below, far lower in ATP concentration than the lowest ATP concentration (0.05 mM) used by Ljones (1973).

We have not determined why some of our enzyme preparations give an induction period before using $S_2O_4^{2-}$ and why some do not. The induction period can be eliminated by preincubation with $MgATP^{2-}$ but the change which ATP accelerates is not known. The temperature dependence of this induction period parallels qualitatively the activation parameters given in Figure 3 and suggests the two effects are related. A possibility is that a definite sequence of substrate addition (an ordered sequence) to the nitrogenase enzyme must occur. For example, if ATP must add first to the enzyme followed by reduction by $S_2O_4^{2-}$, then an induction period might be expected to occur as the enzyme and ATP interact.

Activation Energies. Burns (1969) first reported that nitrogenase from *Azotobacter vinelandii* gave a biphasic Arrhenius plot with a point of intersection at 21 °C. Activation energies of 14.6 and 39 kcal/mol were found above and below this temperature. Similar results were reported by Shilov and Likhtenshtein (1971). Silverstein and Bulen (1970) studied the kinetics of nitrogenase from *Azotobacter vinelandii* as a function of ATP and from their data and the variation of $ATP/2e^-$ reported by Hadfield and Bulen (1969) concluded that two pathways for ATP hydrolysis existed with activation energies differing by 4.6 kcal/mol. One pathway leads to ATP hydrolysis only while the other produces ATP hydrolysis with concomitant electron transfer. Figure 3 confirms the results of Burns (1969) by showing two temperature regions for nitrogenase-catalyzed electron transfer activity with activation energies of 12.3 and 35 kcal/mol. Figure 3 shows further that only a single slope occurs for ATP hydrolysis with an activation energy of 17 kcal/mol. A single straight line could be drawn through the ATP hydrolysis data given by Burns (1969), instead of the two lines he drew, which gives a reasonable fit of his data. This line yields an activation energy of 20 kcal/mol, in fair agreement with our result of 17 kcal/mol from Figure 3. More recently, Thorneley et al. (1975) have determined the temperature dependence of the rate of acetylene reduction by a 1:1 complex of *Klebsiella pneumoniae* Fe protein and FeMo protein and find no break in the resulting Arrhenius plot. An activation energy of +19 kcal/mol results. Our results differ from Thorneley et al. in clearly indicating a sharp break in the Arrhenius plot for the reduction of N_2 and acetylene reduction and for H_2 evolution.

The two processes postulated by Silverstein and Bulen (1970) differing in activation energy by 4.6 kcal/mol in the temperature range 20–40 °C are seen from Figure 3 to be ATP hydrolysis (17 kcal/mol) and electron transfer (12.3 kcal/mol). Because ATP hydrolysis has a larger activation energy than electron transfer, an increase in temperature produces a correspondingly greater increase in the rate of ATP hydrolysis than in the rate of electron transfer to form products. The rate of increase in ATP hydrolysis with temperature over that for electron transfer causes the $ATP/2e^-$ value to increase with temperature as reported by Hadfield and Bulen (1969) and Watt et al. (1975). The molecular basis for these effects is not known at present but protein modification with temperature has been suggested (Watt et al., 1975).

Below 20 °C, a similar situation occurs except that the activation energy for electron transfer is now greater than that for ATP hydrolysis. In fact at 10 °C electron transfer to products is barely detectable, while ATP hydrolysis proceeds at an easily measurable rate. At this temperature, nitrogenase approaches the condition of being simply an ATPase. Because electron transfer is so drastically retarded below 20 °C, while ATP hydrolysis continues, the $ATP/2e^-$ ratio rapidly increases with decreasing temperature as was found by Watt et al. (1975). Again, the molecular basis for these observations is not known but conformational changes affecting electron transfer seem plausible.

Mechanistic Interpretation. The reciprocal plots in Figures 6a and 6b show that the point of intersection on the ordinate is a function only of the $S_2O_4^{2-}$ concentration. At these intercepts the concentration of ATP is infinite and the enzyme is fully saturated with ATP. The rate of the enzyme reaction under these conditions of saturating ATP depends only upon the square root of the $S_2O_4^{2-}$ concentration and as has been previously discussed indicates that SO_2^- is probably the active reductant. The reciprocal plot of Figure 5 shows that variation in the ATP concentration has no effect upon the intercepts, all of which intersect at zero, but its variation does alter the slope of the lines in a manner given by eq 4. This behavior indicates that ATP and enzyme are in thermodynamic equilibrium (Cleland, 1963) and that a change in the ATP concentration specifically alters the net rate of the reaction by altering the concentration of the enzyme–ATP complex which then reacts with SO_2^-. These two kinetic patterns indicate that ATP binding occurs first followed by reduction by SO_2^- as given by the following ordered sequence of events where E and E′ are probably the oxidized and reduced forms of the Fe protein component of nitrogenase, respectively.

$$E + 2ATP \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E(ATP)_2 \quad (5)$$

$$E(ATP)_2 + SO_2^- \xrightarrow{k_2} E'(ATP)_2 + SO_3^{2-} \quad (6)$$

$$E'(ATP)_2 \xrightarrow{k_3} \text{products} + E(ADP)_2 \quad (7)$$

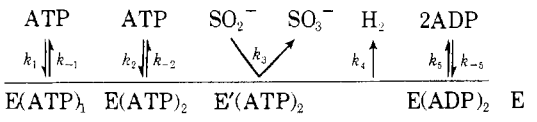
$$E(ADP)_2 \underset{k_5}{\overset{k_4}{\rightleftharpoons}} E + 2ADP \quad (8)$$

Reaction 5 could be written as two successive steps each involving the addition of one ATP because ATP is probably

binding to interacting sites on the protein thus making the two steps kinetically inequivalent. The relationship involving two ATP molecules per SO_2^- given by reactions 5 and 6 follows from the rate law, eq 2, and in addition is supported by: (1) the stoichiometric relationship of ATP/e^- $\cong$ 2 (Watt et al., 1975, and references therein); (2) binding experiments showing two ATP molecules binding to reduced Fe protein (Tso and Burris, 1973); and (3) EPR studies of single electron reduced Fe protein in the presence of ATP (Zumft et al., 1973).

We have written reaction 6 as an irreversible reaction in accord with reasons given above. By analogy to reactions of SO_2^- with redox proteins, some closely related to the proteins of nitrogenase, reaction 6 could have a rate constant as large as 10^7 M^{-1} s^{-1} (Lambeth and Palmer, 1973). Indeed, Thorneley et al. (1976) have found the rate constant for reduction of dye-oxidized Fe protein by SO_2^- to be $>10^8$ M^{-1} s^{-1}. The one-half-order rate constant of 2.3×10^{-3} $M^{1/2}$ ml min^{-1} mg^{-1} determined in this study for $S_2O_4^{2-}$ utilization by nitrogenase can be converted into a value of 3×10^5 M^{-1} s^{-1} for the utilization of SO_2^- by nitrogenase using the equilibrium constant for reaction 3. Though the conditions of the two experiments differ somewhat, it seems reasonable to conclude that the reduction of the Fe protein under turnover conditions is slower by a factor of $>10^3$ than reduction of isolated Fe protein.

The mechanism depicted by reactions 5 to 8 and the above discussion can be summarized by eq 9



(9)

which is of the same form as the so-called Theorell–Chance mechanism (Cleland, 1963) in which no central complex involving both ATP and SO_2^- exists. The general rate equation for this mechanism has been derived (Cleland, 1963) and should contain terms in the denominator involving both $[SO_2^-]$ and $[SO_2^-][ATP]$ in terms of mechanism 9. The rate equation (eq 2) which provides a good fit to our data (see Figure 5) contains no such terms. The reason for their absence may be that the combination of rate constants which makes the coefficients to the $[SO_2^-]$ and $[SO_2^-][ATP]$ terms and the very low concentration of SO_2^- ($[SO_2^-] = (1.4 \times 10^{-9})^{1/2}$-$[S_2O_4^{2-}]^{1/2}$, where 1.4×10^{-9} is the dissociation constant for $S_2O_4^{2-}$) makes these terms small in comparison with the other terms in the denominator of the derived rate equation. The constants $K_1 = 5 \times 10^{-5}$ and $K_2 = 2.8 \times 10^{-9}$ of eq 2 are identified as the dissociation constants for ATP interacting with nonreduced nitrogenase and are in fair agreement with corresponding values reported (Tso and Burris, 1973) for ATP ($K_1 = 1.7 \times 10^{-5}$ and $K_2 = 3 \times 10^{-10}$) interacting with $S_2O_4^{2-}$ reduced Fe protein from *Clostridium pasteurianium*.

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References

Bergersen, F. J., and Turner, G. L. (1973), *Biochem. J. 131,* 61.

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

Bulen, W. A., and LeComte, J. R. (1972), *Methods Enzymol. 24B.*

Burns, R. C. (1969), *Biochim. Biophys. Acta 171,* 253.

Cleland, W. W. (1963), *Biochim. Biophys. Acta 67,* 104.

Creutz, C., and Sutin, N. (1974), *Inorg. Chem. 13,* 2041.

Eady, R. E., and Postgate, J. R. (1974), *Nature (London) 249,* 805.

Evans, M. C. W., and Albrecht, S. L. (1974), *Biochem. Biophys. Res. Commum. 61,* 1187.

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

Lambeth, D. O., and Palmer, G. (1973), *J. Biol. Chem. 248,* 6095.

Ljones, T. (1973), *Biochim. Biophys. Acta 321,* 103.

Ljones, T., and Burris, R. H. (1972), *Biochim. Biophys. Acta 275,* 93.

Mahler, H. R., and Cordes, E. H. (1966), Biochemical Chemistry, New York, N.Y., Harper & Row, p 259.

Meites, L. (1955), Polarographic Techniques, New York, N.Y., Interscience, p 282.

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

Postgate, J. R. (1971), The Chemistry and Biochemistry of Nitrogen Fixation, London, Plenum Press, p 167.

Shilov, A. E., and Likhtenshtein, G. I. (1971), *Ser. Biol., Monogr.-Organ. Los Estados Am. Programa Reg. Desarrollo Cient. Tecnol. No. 4,* 518.

Silverstein, R., and Bulen, W. A. (1970), *Biochemistry 9,* 3809.

Smith, B. E., Lowe, D. J., and Bray, R. C. (1973), *Biochem. J. 135,* 331.

Thorneley, R. N. F., Eady, R. R., and Yates, G. (1975), *Biochim. Biophys. Acta 403,* 269.

Thorneley, R. N. F., and Willison, K. R. (1974), *Biochem. J. 139,* 211.

Thorneley, R. N. F., Yates, G. M., and Lowe, D. (1976), *Biochem. J. 155,* 137.

Tso, M.-Y. W., and Burris, R. H. (1973), *Biochim. Biophys. Acta 309,* 263.

Watt, G. D. (1976), Proceedings of the IInd International Symposium on Nitrogen Fixation, Sept, 1976, Newton, W. E., Postgate, J. R., and Rodriguez-Barrueco, C., Ed., London, Academic Press.

Watt, G. D., and Bulen, W. A. (1974), International Symposium on Nitrogen Fixation, Newton, W. E., and Nyman, C. J., Ed., Pullman, Wash., Washington State University Press.

Watt, G. D., Bulen, W. A., Burns, A., and Hadfield, K. L. (1975), *Biochemistry 14,* 4266.

Yates, M. G., Thorneley, R. N. F., and Lowe, D. J. (1975), *FEBS Lett. 60,* 84.

Zumft, W. G., and Mortenson, L. E. (1975), *Biochem. Biophys. Acta 416,* 1.

Zumft, W. G., Mortenson, L. E., and Palmer, G. (1974), *Eur. J. Biochem. 46,* 525.

Zumft, W. G., Palmer, G., and Morterson, L. E. (1973), *Biochim. Biophys. Acta 292,* 413.