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# Stoichiometry, ATP/2e Values, and Energy Requirements for Reactions Catalyzed by Nitrogenase from *Azotobacter vinelandii*†

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ABSTRACT: The stoichiometry of the nitrogenase ATP-dependent $H_2$ evolution and acetylene reduction reactions using $S_2O_4^{2-}$ as an electron source was studied by various techniques. For each mole of $S_2O_4^{2-}$ oxidized to $2SO_3^{2-}$ by the enzyme-catalyzed reactions at 25° and pH 8, 1 mol of $H_2$ (1 mol of ethylene for acetylene reduction) and two protons are produced. Under these conditions, 4.5 mol of ATP was hydrolyzed to ADP and inorganic phosphate for each $S_2O_4^{2-}$ oxidized. ATP/$S_2O_4^{2-}$ (ATP/2e) values determined at 5° intervals from 10 to 35° were found to go through a minimum at 20°. This effect is explained in terms of possible enzyme structure modifications. Calorimetric measurements for the enzyme-catalyzed $H_2$ evolution and acetylene reduction reactions gave $\Delta H$ values of −32.4 and −75.1 kcal/mol of $S_2O_4^{2-}$, respectively.

The nitrogen reducing enzyme, nitrogenase, from various biological sources is capable of reducing $N_2$ to ammonia when supplied with adenosine 5′-triphosphate (ATP), $Mg^{2+}$, and a source of low potential electrons. Other substrates (Burris, 1971; Murray and Smith, 1968; Hardy and Burns, 1968) including $H^+$ ($H_2O$) are reduced under the same conditions with apparently the same ATP requirement (Hadfield and Bulen, 1969) per reducing equivalent transferred by the enzyme. The amount of ATP required to transfer two electrons, the ATP/2e value, has been a much sought after quantity but reported results still indicate some disagreement. Most reported ATP/2e values fall in the range 4–6 at 25–30° but extreme values of from 2 to 18 have been reported (Hardy and Knight, 1966; Kelly, 1969; Jeng et al., 1970).

The function of ATP is not known but its energy content is likely to be released in some controlled way in satisfying the activation requirements of $N_2$. Consequently, the amount of ATP utilized for electron transfer (ATP/2e) is an essential quantity in determining the total energy necessary for enzymatic $N_2$ reduction. Furthermore, the ATP/2e value determined under various conditions of temperature, pH, etc., may provide insight into the purpose of the ATP requirement as well as the mechanism for its utilization by nitrogenase.

As a prelude to determining both the energetics of nitrogenase action and the energy requirements of each of the nitrogenase component proteins, it was necessary to determine precisely the ATP/2e value, the overall stoichiometry, and the thermodynamic states of each reactant and product. The results of these experiments and the energy changes accompanying them are the subject of this report.

## Experimental Section

*Materials.* Adenosine 5′-triphosphate (ATP) and *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid

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(TES)[1] 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 Companies Ltd., Harrogate, Yorkshire, England. The sealed metal container was opened and subsequently stored in a Vacuum Atmospheres Corporation drybox under oxygen-free argon. Smaller samples of $Na_2S_2O_4$ for laboratory use were removed from 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 riboflavine 5′-phosphate. Argon and prepurified nitrogen were passed through a hot quartz tube (600°) containing copper turnings to remove oxygen prior to use. Preparations of the nitrogenase complex from *Azotobacter vinelandii* with specific activities of 200–300 nmol of $H_2$ per min per mg of complex were obtained as previously described (Bulen and LeComte, 1966, 1972; W. A. Bulen and J. R. LeComte, unpublished results).

*Methods:* All solutions were made 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 chromatograph (molecular sieve 5A, argon carrier gas). Ethylene formation was also determined by chromatography on alumina.

A creatine phosphate–creatine kinase generating system was used to maintain a constant ATP concentration and to prevent ADP from reaching inhibitory levels. The $P_i$ released was measured as previously described (Bulen and LeComte, 1966) using controls minus $Na_2S_2O_4$.

*Instrumental Procedures.* (a) pH-Stat Methods. Changes in $H^+$ concentration during the enzyme-catalyzed $H_2$ evolution reaction were measured with a Radiometer Model TTT1C pH-Stat. The procedure consisted of placing 2.0 ml of an anaerobic solution containing 1–5 mg of nitrogenase, 0.2–0.8 μmol of $Mg^{2+}$, and 0.4–1.6 μmol of ATP in the anaerobic titration cup of the pH-Stat and adjusting the pH to 8.00. When equilibrated, excess solid $Na_2S_2O_4$ or a solution of $S_2O_4^{2-}$ in $H_2O$ at pH 8.00 was added to initiate the reaction. The amount of 0.01 *M* NaOH required to maintain pH 8.00 was then measured as the known volume of a standard ATP solution was hydrolyzed by the enzyme-catalyzed reaction. Control reactions consisted of 2.0 ml of solution containing enzyme but minus ATP and $Mg^{2+}$.

Another set of experiments was performed as above except that the ATP-generating system was included to keep the ATP concentration constant in the 2.0-ml volume and a known amount of $S_2O_4^{2-}$ was allowed to react. The $S_2O_4^{2-}$ solution was standardized amperometrically just prior to use (see below). These experiments yielded the $H^+$/ATP and the $H^+/S_2O_4^{2-}$ values, respectively.

(b) Polarographic Methods. Total $S_2O_4^{2-}$ utilization by the nitrogenase-catalyzed reaction was measured amperometrically using a Sargent Model XXI polarograph. The polarograph was equipped with a dropping mercury electrode (DME) connected to a 10-ml sample compartment of an "H-cell". The other compartment contained a saturated calomel electrode (SCE) attached through a medium glass frit to the sample side. The H-cell had copper tubing gas lines connected to the sample compartment so that the sample area could be flushed with oxygen-free argon or nitrogen prior to filling and also for maintaining an oxygen-free atmosphere above the enzyme solution during the measurements. The H-cell was thermostated (±0.05°) in a vibration-free water bath.

The polarograph and cell were calibrated to relate anodic diffusion current to $S_2O_4^{2-}$ concentration at the various temperatures, cell volumes, and buffer components used by titrating the assay mixture containing enzyme, generating system minus ATP, and $S_2O_4^{2-}$ with standard $Fe(CN)_6^{3-}$ or ferricytochrome *c* solutions. This calibration procedure allowed a rapid and precise determination of $S_2O_4^{2-}$ concentration at any given time by recording its anodic diffusion current.

The procedure for measuring the ATP/2e value or the rate of $S_2O_4^{2-}$ utilization by nitrogenase was as follows. An anaerobic 2.00–5.00-ml assay sample containing 200–300 μmol of TES or Tris buffer, 260 μmol of creatine phosphate, 5 nmol of creatine kinase, 2–8 μmol of ATP, and 1–4 μmol of $MgCl_2$ at pH 7.0 or 8.0 was placed in the anaerobic sample compartment of the H-cell containing the DME. Solid $Na_2S_2O_4$ (1–10 μmol) was added to the assay solution in the sample compartment of the H-cell, stirred briefly by a magnetic stirrer, and then the DME was activated. The voltage of the DME was set at +0.30 V (a plateau of the $S_2O_4^{2-}$ half-wave centered at +0.50 V) relative to the SCE and the anodic diffusion current corresponding to the $S_2O_4^{2-}$ concentration was recorded. Enzyme was added, the mixture briefly stirred, and then the anodic diffusion current of $S_2O_4^{2-}$ was continuously recorded as the enzyme-catalyzed utilization of $S_2O_4^{2-}$ proceeded. The reactions were usually allowed to continue until the enzyme-catalyzed reaction exhausted the $S_2O_4^{2-}$. In some cases, the reactions were stopped at selected stages of reaction by adding excess $Fe(CN)_6^{3-}$ which oxidized all remaining $S_2O_4^{2-}$ and stopped the enzyme-catalyzed reaction. The reactions were complete in 10–20 min except at 10–15° where they were monitored for 40–50 min. When the reaction was completed several 0.50-ml samples of solution were removed for phosphate analysis and in those reactions run in a closed cell, gas samples were removed and analyzed. Control reactions to determine phosphate blanks contained all reagents except $S_2O_4^{2-}$. Two other controls were run consisting of: (1) the 5.00-ml assay mixture as above, but minus enzyme and (2) the 5.00-ml assay mixture containing everything but ATP. These controls determined $S_2O_4^{2-}$ utilization in the absence of enzymic catalysis and showed <1% change in $S_2O_4^{2-}$ concentration during the reaction interval. This allows us to confidently attribute phosphate production and $S_2O_4^{2-}$ utilization to the enzyme-catalyzed reaction only. In some experiments, substrate levels of ATP were added without a generating system present and the amount of $S_2O_4^{2-}$ required to hydrolyze this known amount of ATP was measured.

ATP/2e values were also determined at 10 and 15° by a manometric procedure previously described (Hadfield and Bulen, 1969; Bulen and LeComte, 1966). At these low temperatures the rate of hydrogen production was too slow to

[1] Abbreviations used are: TES, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid; DME, dropping mercury electrode; SCE, saturated calomel electrode; CP, creatine phosphate; Fe protein and Mo–Fe protein, the two-component proteins of nitrogenase containing iron only and iron and molybdenum, respectively; $P_i$, phosphate released from ATP hydrolysis.

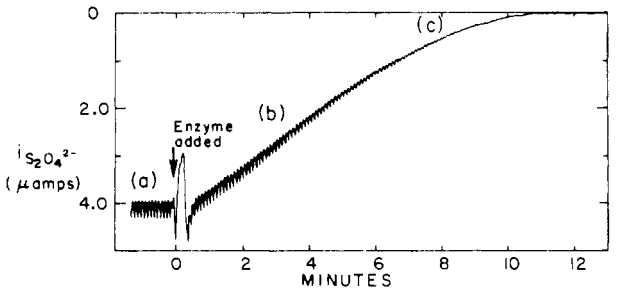


FIGURE 1: A polarographic trace of the nitrogenase-catalyzed utilization of $S_2O_4^{2-}$ during the $H_2$ evolution reaction. The ordinate is the anodic diffusion current of $S_2O_4^{2-}$ and is directly proportional to $S_2O_4^{2-}$ concentration ($[S_2O_4^{2-}] = 1.375 \times 10^{-4}\, i_{S_2O_4^{2-}}$). 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).

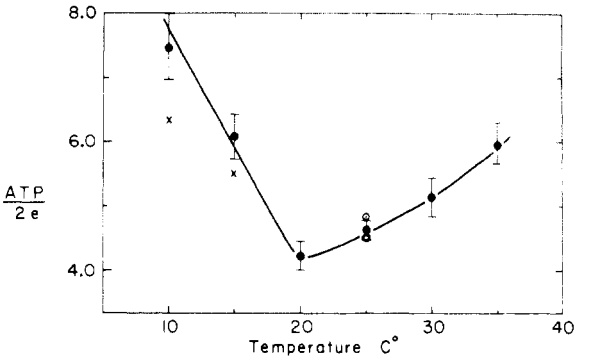


FIGURE 2: The variation of the ATP/2e value with temperature. (I) Polarographically determined; (O) calorimetrically determined; (●) determined from pH-Stat measurements; (x) manometrically determined.

allow precise ATP/2e values to be determined manometrically and the polarographically determined values are preferred.

(c) Calorimetric Measurements. A Beckman Model 190-C microflow calorimeter of the type previously described (Sturtevant and Lyons, 1969) was used for the enthalpy measurements. The Teflon flow lines were coaxially jacketed with larger diameter Teflon lines through which purified argon or nitrogen flowed. This isolated and maintained anaerobic conditions in the flow lines containing the oxygen-sensitive nitrogenase and $S_2O_4^{2-}$ solutions. *Teflon "spaghetti" tubing is sufficiently permeable to oxygen that significant nitrogenase or $S_2O_4^{2-}$ oxidation can occur if the above precaution is not observed.*

Three types of calorimetric measurements were performed. First, enthalpy measurements of the hydrogen evolution reaction using limiting substrate levels of ATP and excess $S_2O_4^{2-}$. Several alternative methods of mixing the various components were tried but the combination of mixing enzyme and $S_2O_4^{2-}$ in buffer carefully adjusted to pH 7.50 or 8.00 with $S_2O_4^{2-}$ and ATP-$Mg^{2+}$ in buffer adjusted to pH 7.50 or 8.00 gave the most reliable and reproducible results. The following thermal corrections were required: (1) the interaction of released protons with buffer components ($HTris^+$, $\Delta H_{ion} = +11.3$ kcal/mol; $HTES^+$, $\Delta H_{ion} = +8.00$ kcal/mol; and $HSO_3^-$, $\Delta H_{ion} = +6.41$ kcal/mol); (2) the conversion of $Mg^{2+}$-ATP initially present to $Mg^{2+}$-ADP-$P_i$ after reaction; and (3) the interaction of $Mg^{2+}$-ADP-$P_i$ with reduced enzyme. The corrections were measured in separate experiments which duplicated the conditions of the $\Delta H$ measurements.

The second type of calorimetric measurement utilized the ATP-generating system and a limiting concentration of $S_2O_4^{2-}$ standardized amperometrically in the calorimeter. Heat of dilution and buffer ionization corrections were applied.

The third type of measurements was identical with the second type except that the solutions were equilibrated with 0.3–1 atm of acetylene prior to reaction in the calorimeter.

## Results

Figure 1 is a polarographic trace of $S_2O_4^{2-}$ utilization catalyzed by the nitrogenase enzyme under the conditions where enzyme is added last to the assay solution. The various regions (a), (b), and (c) will be dealt with more completely in a following article. Of interest here is the anodic diffusion current, $i_{S_2O_4^{2-}}$, which is proportional to the concentration of $S_2O_4^{2-}$ at any point along the curve.

*ATP/2e Values.* The $i_{S_2O_4^{2-}}$ value at (a) gives the total amount of $S_2O_4^{2-}$ initially present in the assay mixture and therefore the amount consumed during the enzyme-catalyzed reaction. The $S_2O_4^{2-}$ consumption at any point during the reaction can be easily obtained from the curve after correcting for dilution resulting from enzyme or reagent addition. ATP/2e values were calculated by measuring the $P_i$ produced during a measured $S_2O_4^{2-}$ decrease. The results are shown in Figure 2 for several temperatures. There was no significant variation in these ATP/2e values when the reaction was stopped at various times along the curve, or when the pH was varied from 8.0 to 7.0. Occasionally, an enzyme preparation gave ATP/2e values larger than those shown. However, the resulting curve is simply displaced upward but still parallels that shown in Figure 2. The freezing and storage of the enzyme in liquid nitrogen for periods greater than a week tended to cause the ATP/2e values to increase and consequently all results were obtained using enzyme samples which were freshly prepared. While ATP/2e values of 4.6 at 25° were obtained from a freshly prepared enzyme sample, values of 5.3 and 5.8 resulted from the same enzyme sample which had been frozen and stored in liquid nitrogen for 14 and 30 days, respectively.

In some experiments, a temperature-sensitive induction period was observed preceding region (b) of Figure 1 in which no $S_2O_4^{2-}$ utilization occurred even though all components necessary for nitrogenase-catalyzed $H_2$ evolution were present. After 0.5–1 min at 20° (longer times at lower temperatures, shorter times at higher temperatures), reaction began and region (b) appeared followed by region (c). The presence of this induction period did not alter the ATP/2e value nor the rate at which $S_2O_4^{2-}$ was used in regions (b) and (c).

*Proton Release Measurements.* When limiting substrate levels of ATP are hydrolyzed at 25.0° by the nitrogenase-catalyzed hydrogen evolution reaction in the pH-Stat, 1.41 ± 0.06 $H^+$ are produced per ATP hydrolyzed at pH 8.0. As both ATP hydrolysis and $S_2O_4^{2-}$ oxidation at pH 8.0 under the conditions of our experiments are linked to $H^+$ production as shown by reactions 1 and 2, respectively,

$$ATP \overset{H_2O}{\rightleftarrows} ADP + P_i + H^+ \quad (1)$$

$$S_2O_4^{2-} + 2H_2O \rightleftarrows 2SO_3^{2-} + H_2 + 2H^+ \quad (2)$$

an ATP/$S_2O_4^{2-}$ (ATP/2e) value of 4.8 ± 0.5 can be calcu-

Table I: $\Delta H$ Values at 25° for the Nitrogenase-Catalyzed $H_2$ Evolution Reaction Using Substrate Levels of ATP.

| Expt | pH | Buffer | $\Delta H_{obsd}$ (kcal/mol of ATP) | $\Delta H_{corr}$ (kcal/mol of ATP) | $\Delta H_{corr}$ (kcal/mol of $S_2O_4^{2-}$) |
|---|---|---|---|---|---|
| 1 | 7.5 | Tris | −10.6 ± 0.5 | −2.5 ± 0.5 | −11.8 ± 2.4 |
| 2 | 7.5 | Tris | −11.4 ± 0.3 | −3.3 ± 0.3 | −15.5 ± 1.4 |
| 3 | 7.5 | Tris | −10.0 ± 0.6 | −2.8 ± 0.6 | −13.7 ± 2.8 |
| 4 | 8.0 | Tris | −16.9 ± 0.4 | −3.4 ± 0.4 | −15.7 ± 1.8 |
| 5 | 8.0 | TES | −13.5 ± 0.3 | −3.1 ± 0.3 | −14.6 ± 1.4 |
| | | | | Av −3.1 ± 0.4 | Av −14.2 ± 1.8 |

Table II: $\Delta H$ Values at 25° in TES Buffer (pH 8.0) for the Nitrogenase-Catalyzed $H_2$ Evolution and Acetylene Reduction Reactions in the Presence of an ATP Generating System and Limiting $S_2O_4^{2-}$.

| Expt | Atmosphere | $\Delta H_{obsd}$ (kcal/$S_2O_4^{2-}$) | $\Delta H_{corr}$ (kcal/$S_2O_4^{2-}$) |
|---|---|---|---|
| 1 | Argon | −47.6 | −32.6 |
| 2 | Argon | −46.9 | −31.9 |
| 3 | Argon | −46.5 | −31.5 |
| 4 | Argon | −47.5 | −32.5 |
| 5 | Argon | −48.3 | −33.5 |
| | Av | −47.4 ± 0.9 | Av −32.4 ± 0.9 |
| 6 | Acetylene | −92.6 | −77.6 |
| 7 | Acetylene | −89.2 | −74.2 |
| 8 | Acetylene | −88.5 | −73.5 |
| | Av | −90.1 ± 2.5 | Av −75.1 ± 2.5 |

lated from the $H^+$/ATP value given above. The ATP/2e value at 25° thus calculated is in reasonable agreement with that given in Figure 2 but the experimental uncertainty is slightly larger by this method.

At pH 8.0 and 25°, the oxidation of limiting levels of amperometrically standardized $S_2O_4^{2-}$ by the nitrogenase-catalyzed $H_2$ evolution reaction in the presence of an ATP generating system produced 1.96 ± 0.04 protons per $S_2O_4^{2-}$ oxidized. Under these conditions, the hydrolysis of creatine phosphate via creatine kinase maintains a constant ATP concentration by converting ADP back into ATP, and as no protons are exchanged during phosphate hydrolysis, there is no net change in $[H^+]$ produced by the running of the generating system (Fruton and Simmonds, 1958). Because there is no proton link between $S_2O_4^{2-}$ oxidation and $P_i$ production under these conditions, an ATP/2e value cannot be calculated. These data show, however, that 1 mol of hydrogen and two protons are produced per $S_2O_4^{2-}$ oxidized and provide the necessary information to be used for buffer ionization corrections of the calorimetric data discussed next.

*Calorimetry.* Table I contains calorimetric data for the nitrogenase-catalyzed $H_2$ evolution reaction in which limiting amounts of ATP were hydrolyzed in the calorimeter at pH values of 7.5 and 8.0. The error limits are fairly large because of the small amounts of heat produced and because as the ADP builds up, the reaction becomes inhibited and tails off to completion. From the measured $\Delta H$ value of −14.2 kcal/mol of $S_2O_4^{2-}$ for the nitrogenase-catalyzed $H_2$ evolution reaction and a $\Delta H$ value of +7.2 kcal/mol of $S_2O_4^{2-}$ (Watt and Burns, 1975) for reaction 2, a $\Delta H$ value of −4.7 ± 0.5 kcal/mol is calculated for the enthalpy of hydrolysis of ATP under these experimental conditions. This value is slightly low, probably because of incomplete reaction due to ADP inhibition, but is in reasonable agreement with published $\Delta H$ values (Podolsky and Sturtevant, 1955; Podolsky and Morales, 1956; Cereijo-Santalo, 1971; Raison and Evans, 1968) of −4.7 to −6.2 kcal.mol for the enthalpy of hydrolysis of ATP and confirms the data in Table I.

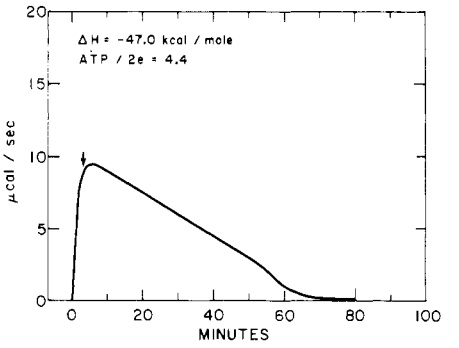


FIGURE 3: A calorimetric trace of the nitrogenase-catalyzed $H_2$ evolution reaction at 25° and in pH 8.0 Tes buffer. The reaction mixture contained the ATP-generating system and a known, limiting amount of $S_2O_4^{2-}$ and was allowed to react in the calorimeter until all of the $S_2O_4^{2-}$ was consumed. The arrow indicates the point where the flow to the calorimeter was stopped.

In Table II are reported $\Delta H$ values per mole of $S_2O_4^{2-}$ for the nitrogenase-catalyzed $H_2$ evolution (−32.4 kcal/mol) and acetylene reduction reactions (−75.1 kcal/mol) in the presence of an ATP-generating system with limiting $S_2O_4^{2-}$. The calorimeter trace for the $H_2$ evolution reaction is shown in Figure 3. The ATP-generating system eliminates the problem of ADP inhibition discussed above but substitutes creatine phosphate hydrolysis for ATP hydrolysis. The two reactions are described by (3) and (4), respectively:

$$S_2O_4^{2-} + 2H_2O + n\text{CP} \rightleftarrows 2SO_3^{2-} + H_2 + 2H^+ + n\text{C} + n\text{P}_i \quad (3)$$

$$\Delta H = -32.4 \text{ kcal/mol}$$

$$S_2O_4^{2-} + 2H_2O + \text{HC}{\equiv}\text{CH} + n\text{CP} \rightleftarrows 2SO_3^{2-} + \text{H}_2\text{C}{=}\text{CH}_2 + 2H^+ + n\text{C} + n\text{P}_i \quad (4)$$

$$\Delta H = -75.1 \text{ kcal/mol}$$

where $n$ is the number of moles of creatine phosphate (CP) hydrolyzed per mole of $S_2O_4^{2-}$ oxidized. According to Figure 2, this $n$ value at 25° should be equal to 4.6. We have left $n$ unspecified, however, because it can be determined independently from the data in Table II using the heat balance for reactions 3 and 4 in the following way. The $\Delta H$ values given in Table II for reaction 3 are expressed per mole of $S_2O_4^{2-}$ oxidized but the number of moles of creatine phosphate hydrolyzed is unknown. If reaction 2 and its $\Delta H$ value of +7.2 kcal/mol of $S_2O_4^{2-}$ (Table III) is substracted from (3), reaction 5 results with a $\Delta H$ value of −39.6 kcal/mol:

$$n\text{CP} = n\text{C} + n\text{P}_i \quad (5)$$

$$\Delta H = n\,(\Delta H \text{ hydrolysis of CP}) = -39.6 \text{ kcal/mol}$$

When reaction 5, for an unspecified number of moles of CP hydrolyzed, is divided by the known (Gallert and Sturtevant, 1960) $\Delta H$ value of −9.0 kcal/mol for hydrolysis of 1 mol of CP, an $n$ value of 4.40 is calculated. This value is in good agreement with the data given in Figure 2. A similar approach can be used for reaction 4, the reduction of acety-

Table III: Thermodynamics of Nitrogenase Substrate Reactions at 25°.

| Reactions[a] | $\Delta H^\circ$ (kcal/mol) | $\Delta G^\circ$ (kcal/mol) |
|---|---|---|
| $ATP \xrightarrow{H_2O} ADP + P_i + H^+(aq)$ | −4.7 to −6.2 | −9.8[b] |
| $2H_2O + S_2O_4^{2-} \rightleftharpoons H_2(g) + 2SO_3^{2-} + 2H^+(aq)$ | +7.2[c] | −34.10 |
| $N_2(g) + 3H_2(g) \rightleftharpoons 2NH_3(aq)$ | −38.64 | −12.76 |
| $N_2(g) + 3H_2(g) + 2H^+(aq) \rightleftharpoons 2NH_4^+(aq)$ | −51.06 | −25.36 |
| $CO(g) + 3H_2(g) \rightleftharpoons CH_4(g) + H_2O(l)$ | −59.78[d] | −36.02 |
| $HCN(aq) + 3H_2(g) + H^+(aq) \rightleftharpoons CH_4(g) + NH_4^+(aq)$ | −75.12 | −58.15 |
| $HC{\equiv}CH(g) + H_2(g) \rightleftharpoons CH_2{=}CH_2(g)$ | −41.69 | −33.72 |
| $HN_3(aq) + H_2(g) \rightleftharpoons N_2(g) + NH_4^+(aq)$ | −83.64 | −96.32 |
| $N_2O(g) + H_2(g) \rightleftharpoons N_2(g) + H_2O(l)$ | −87.81 | −81.45 |

[a] Unless specified otherwise, the data in this table were taken from: NBS Circular 500, Selected Values of Chemical Thermodynamic Properties. [b] Alberty (1968). [c] Watt and Burns (1975). [d] CO is not a substrate of nitrogenase but is an inhibitor of all reactions except $H_2$ evolution.

lene by nitrogenase, giving an $n$ value of 4.50. This calorimetric method is faster, more convenient, and is more precise than the other methods employed in this study for determining ATP/2e values.

The shape of the curve in Figure 3 can be analyzed to provide additional information about the enzyme-catalyzed reactions. This curve was obtained under conditions where a limiting amount of $S_2O_4^{2-}$ reacted in the presence of a constant concentration of ATP provided by the ATP-generating system. As the nitrogenase-catalyzed reaction proceeds, the ATP remains constant but the $S_2O_4^{2-}$ concentration decreases in a manner dependent upon its concentration and the rate law governing its utilization by the enzyme. Heat is produced at a rate which parallels the disappearance of $S_2O_4^{2-}$ oxidation. It is clear from Figure 3 that two well-defined regions of heat production exist after the syringe drive is stopped. One region shows heat production decreasing linearly with time and the second, near the end of the reaction, where heat production asymptotically approaches zero. The linear region of the curve accounts for 90% of the heat production as the $S_2O_4^{2-}$ concentration decreases from its initial concentration of about 5 m$M$ to below 0.1 m$M$. As $S_2O_4^{2-}$ becomes limiting in the presence of a constant ATP concentration, the rate law apparently changes and produces the second region of the curve. A similar result is found for the acetylene reduction reaction.

## Discussion

All three independent methods used in this study to measure ATP/2e values gave results in agreement with each other and in agreement with those published values (Winter and Burris, 1968; Kennedy et al., 1968; Hadfield and Bulen, 1969) in the range of 4–5 at 25–30°. The enzyme preparations used in the course of these studies were obtained by several different enzyme isolation procedures but none of these gave ATP/2e values below those reported in Figure 2. This invariance of the ATP/2e value with variation in isolation procedure indicates that the enzyme preparations are not being altered by the isolation procedures and producing ATP hydrolysis artifacts. The data in Figure 2 consequently represent the minimum number of ATP molecules required to transfer two electrons at a given temperature by the nitrogenase enzyme. We have found, however, that rarely and unpredictably an enzyme preparation can give values higher than but parallel with those reported in Figure 2. The reason for these high value preparations is not completely known but seems to arise from at least two factors. Though difficult to quantitate, we have generally found that enzyme samples exposed briefly to oxygen but not inactivated gave higher ATP/2e values than unexposed samples. Also, high ATP/2e values could be induced in an enzyme sample that normally gave correct ATP/2e values by extended storage in liquid nitrogen or by repeated freezing and thawing without altering any of the other enzyme characteristics (activity, spectra, molecular weight, etc.).

Sensitivity to both cold and oxygen are well-known characteristics of the isolated Fe protein in nitrogenase and it seems likely that the high ATP/2e values are a result of altered or partially inactivated Fe protein. This conclusion is consistent with the work of Tso and Burris (1973), Orme-Johnson et al. (1972), Smith et al. (1973), and Zumft et al. (1974) who have implicated the Fe protein as the ATP binding site of the nitrogenase enzyme. Even more relevant to this conclusion are the reports (Ljones and Burris, 1972; Eady and Postgate, 1974) that inhibition of substrate reduction but not ATP hydrolysis occurs with an artificially created excess of Mo–Fe protein over the Fe protein. The result is an increase in the ATP/2e value with an increasing Mo–Fe protein/Fe protein ratio. In our experiments exposure of the nitrogenase complex to oxygen or excessive cold treatment selectively destroys or inactivates the Fe protein thereby increasing the Fe–Mo protein/Fe protein ratio. The consequence is, as we have observed, an elevated ATP/2e value.

No variation was found in the measured ATP/2e values when the pH was changed from 8.0 to 7.0 or when the total amount of $S_2O_4^{2-}$ used, under otherwise identical conditions, was decreased by a factor of 5. It was found, however, as has been previously reported (Hadfield and Bulen, 1969), that the ATP/2e value is sensitive to temperature change as shown in Figure 2. Hadfield and Bulen (1969) previously reported ATP/2e variation in the temperature range 20–40° and indicated the lack of whole number stoichiometry for ATP hydrolysis. Our results, obtained by several independent methods, confirm their results and show further the dramatic increase in the amount of ATP hydrolysis required to transfer electrons in the temperature region below 20°.

The reason for this variation in ATP/2e with temperature is not clear but it seems likely that temperature-induced alterations in the protein structure of either Fe protein or Mo–Fe protein may be responsible. Below 20°, the protein(s) may be "frozen" into a conformation which is inactive toward electron transfer but is still capable of ATP hydrolysis, thus making ATP/2e increase. Only when sufficient energy has been supplied by ATP hydrolysis does the "frozen" form "melt" and become active in electron transfer. In support of this view, we have found a biphasic activation energy profile (G. D. Watt, unpublished results) for the nitrogenase-catalyzed $H_2$-evolution reaction similar to that reported by Burns (1969) in which the apparent activation energy is +39 and +14 kcal/mol in the temperature range 10–20 and 20–40°, respectively. These observations suggest that the larger activation energy requirement below 20° is being met by energy supplied from increased ATP hydrolysis per 2e transferred by the enzyme. Recalling the cold lability of isolated protein and the ATP-binding ability of this

protein, the conclusion seems warranted that the Fe protein is the component of the nitrogenase complex responding to the temperature variation below 20°.

$$\text{Fe protein} \underset{\text{above } 20°}{\overset{\text{below } 20°}{\rightleftarrows}} \text{Fe protein*} \quad (6)$$

The equilibrium of reaction 6 summarizes the above observations and conclusion by indicating that the active Fe protein can be reversibly converted into an inactive form designated as Fe protein* by lowering the temperature. Reconversion into the active Fe protein occurs either by raising the temperature or through increased ATP hydrolysis. The difference in activation energy of 25 kcal/mol between the two forms gives an indication of the extent of protein modification. Reaction 6 also implies that a lowering of the temperature and consequently a shift of the equilibrium to the right has the effect of increasing the Mo-Fe protein/Fe protein ratio. As discussed above, this increase causes higher ATP/2e values and therefore this effect may also contribute significantly to the increased ATP requirement at lower temperatures. The extent of this effect cannot at present be determined because the equilibrium constant for (6) is not known.

The modest increase in ATP/2e with increasing temperature above 20° may also result from protein structure modifications. Also, if an association equilibrium between Fe protein and Mo-Fe protein is required for enzymic activity as suggested by Silverstein and Bulen (1970) and Thornley (1974) from kinetic studies of nitrogenase catalysis, the increasing temperature would favor dissociation and consequently this "uncoupling" would require more ATP. Further work is clearly required particularly in demonstrating more definitely the nature and extent of these postulated protein structure modifications with temperature.

It would also be desirable to study nitrogenases from other organisms over the temperature range covered in this study to determine whether similar temperature effects would be observed. Is the strong association of the Fe and Mo-Fe proteins of *Azotobacter* nitrogenase somehow responsible for the temperature effects observed or would similar results be obtained from the easily dissociable nitrogenase from *Clostridium pasteuranium?*

The minimum in Figure 2 of 4.2 at 20° might seem to be simply a fortuitious result of the low temperature effect and the high temperature effect combining to produce a minimum numerically equal to 4.2 at 20°. However, the very sharp break in the activation energy curve (Burns, 1969 and our unpublished results) near 20° and the relative slopes of the two lines of Figure 2 argue that a blending of two temperature effects over as wide a range as would be required to produce a "composite" curve like that of Figure 2 is not occurring. We believe that the low and high temperature effects near 20° are minor contributions to a fundamental enzymatic process with a whole number ATP/2e value of 4.0. As the high (>20°) and low temperature (<20°) effects become important, deviations from this pure stoichiometry occur because ATP hydrolysis continues but apparently without accompanying electron transfer. If this view is correct, the hydrolysis of two ATP's is required to transfer one electron by the nitrogenase components but as the protein conformations change with temperatures less efficient use of ATP occurs. The binding of two ATP molecules by the one electron reduced Fe protein reported by Tso and Burris (1973) is consistent with this stoichiometry and implies that the bound ATP molecules are hydrolyzed upon transfer of the electron from this protein.

From the proton release measurements of the nitrogenase-catalyzed $H_2$ evolution reaction, it is clear that 1 mol of hydrogen is produced/mol of $S_2O_4^{2-}$ oxidized by the enzyme. This same result is obtained indirectly from the identical ATP/$S_2O_4^{2-}$ and ATP/$H_2$ values measured independently by polarographic and previously reported manometric methods (Hadfield and Bulen, 1969), respectively. These results were also confirmed in one experiment by monitoring $S_2O_4^{2-}$ utilization, $H_2$ production, $P_i$ production, and $H^+$ liberation. These experiments show unequivocally that the nitrogenase-catalyzed $H_2$ evolution reaction at pH 8.0 or above obeys the stoichiometry of reaction 3, where $n$ is the temperature-dependent number of ATP molecules required as found in Figure 2. This extensive study of the stoichiometry is essential in preparing for detailed thermodynamic studies of other nitrogenase-catalyzed reactions and for thermodynamic studies of the nitrogenase complex itself as well as its constituent proteins.

The stoichiometry for acetylene reduction at pH 8.0 and 0.3-1.0 atm of acetylene catalyzed by nitrogenase was found, as expected, to obey reaction 7. Reaction 7 is simply reaction 3 at 25° ($n$ = 4.6) to which acetylene reduction by molecular hydrogen has been added. The addition of substrate reduction by $H_2$ to reaction 3 can be generalized to calculate the stoichiometry or thermodynamics for the reduction of any other substrate reducible by nitrogenase after correcting for any accompanying $H_2$ evolution reaction.

$$\text{H—C}\equiv\text{C—H} + S_2O_4^{2-} + 2H_2O + 4.6\text{ATP} \rightleftarrows H_2C{=}CH_2 + 2SO_3 + 6.6H^+ + 4.6P_i + 4.6\text{ADP} \quad (7)$$

Below pH 8.0, the stoichiometry of (3) becomes more complicated because proton liberation from ATP hydrolysis becomes fractional (Alberty, 1968) as it decreases from one to near zero as a function of decreasing pH. Also, the apparent proton release from $S_2O_4^{2-}$ decreases due to $HSO_3^-$ formation in accord with its p$K$ of 6.9. The complexity introduced by these two processes plus the fact that $S_2O_4^{2-}$ becomes less stable with decreasing pH (Lem and Wayman, 1970) is great enough to make interpretation of results below pH 8.0 too difficult to be worthwhile and consequently most measurements reported here were done at pH 8.0.

From a thermochemical point of view, reaction 3, the nitrogenase-catalyzed hydrogen evolution reaction, can be considered to be the sum of the isolated reactions 1 and 2. Table III contains thermochemical data for these two reactions plus data for the reduction by hydrogen of other substrates of nitrogenase. It is clear that $S_2O_4^{2-}$ supplies more than enough energy to produce hydrogen which when produced is capable (thermodynamically but not necessarily kinetically) of reducing all of the known substrates of nitrogenase. Other electron donors for nitrogenase include methyl viologen, ferredoxin, and flavodoxin all with potentials near or more negative than the pH 7 hydrogen electrode (−430 mV). A recent report from this laboratory (Watt and Bulen, 1975) has shown that a voltage more negative than −400 mV is required to sustain nitrogen fixation by nitrogenase from *A. vinelandii*. It appears then that none of the energy supplied by ATP is necessary thermodynamically to drive the reactions all of which should proceed spontaneously without it. This is verified calorimetrically because the energy beyond that needed thermodynamically by the

reaction is fully accounted for as liberated heat of reaction. The ATP energy is undoubtedly used in making reactive energy-containing enzyme intermediates but as the enzyme is turning over, i.e., alternating between intermediates and native enzyme, no information regarding the energy content of these intermediates can be gained from the thermodynamics of the overall enzyme-catalyzed reactions reported in Tables I and II. Information regarding the energy content of enzyme intermediates must be gained from thermodynamic studies of the constituent proteins of nitrogenase under various conditions and these studies are presently underway in our laboratory.

The information found in Tables I and II combined with the ATP stoichiometry clearly delineated in this study correspond to a single enzyme turnover. When the thermodynamics for easily obtained enzyme intermediates (i.e., reduced Mo-Fe protein, reduced Fe protein, the binding of ATP to Fe protein, etc.) are substracted from the data for a single turnover, data for difficult to obtain or unknown enzyme intermediates can be inferred.

The kinetics of utilization of $S_2O_4^{2-}$ by nitrogenase monitored by the rate of heat production with time is an interesting and unique application of calorimetry to studies of nitrogenase-catalyzed reactions. These measurements show that under the conditions of constant ATP concentration and limiting $S_2O_4^{2-}$, the rate dependence on the $S_2O_4^{2-}$ concentration becomes half-order for both nitrogenase-catalyzed $H_2$ evolution and acetylene reduction. This half-order dependence may indicate a rate-limiting one-electron transfer to the enzyme from the two-electron $S_2O_4^{2-}$ donor. More information concerning the kinetics of nitrogenase-catalyzed reactions will appear in a later publication.

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