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Adenosine Triphosphate Requirement of Nitrogenase from *Azotobacter vinelandii**

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ABSTRACT: Knowledge of the amount of adenosine triphosphate hydrolyzed during the transfer of reducing electrons in the reactions catalyzed by nitrogenase (the adenosine triphosphate:$2e^-$ ratio) is requisite to understanding the energetics of the reactions and perhaps also the function(s) of adenosine triphosphate. Most reported adenosine triphosphate:$2e^-$ ratios range between 2 and 5, but both higher and lower values have been indicated. An extensive examination of adenosine triphosphate hydrolysis in the H_2 evolution and N_2 or C_2H_2 reductions catalyzed by the nitrogenase complex from *Azotobacter vinelandii* gave ratios approaching 5 at 30°. This ratio was independent of substrate, substrate concentration, H_2 inhibition, and pH but was temperature dependent.

Adenosine triphosphate:$2e^-$ values increased with temperature from 4.3 at 20° to 5.8 at 40°. The discussion includes an attempt to explain variations in the values observed with the clostridial and azotobacter enzymes. It is suggested that adenosine triphosphate is hydrolyzed in two ways, only one of which leads to electron transfer and that the concept of single-reaction stoichiometry is not applicable to this system.

A requirement for ATP in the reactions catalyzed by nitrogenase, the N_2-reducing enzyme complex, was suggested by arsenate and glucose inhibition experiments (McNary and Burris, 1962) and was subsequently established with *Clostridium pasteurianum* (Mortenson, 1964; Hardy and D'Eustachio, 1964) and *Azotobacter vinelandii* preparations (Bulen *et al.*, 1964). In addition to ATP, a source of electrons, such as dithionite (Bulen *et al.*, 1965) or enzyme systems providing reduced ferredoxin (Mortenson, 1964), is required for the reduction of N_2 or alternate substrates (N_3^-, N_2O, C_2H_2, CN^-, CH_3NC, etc.). As with other ATP-requiring enzymes, a divalent metal, preferably Mg^{2+}, is required (Burns and Bulen, 1965). The utilization of ATP is not restricted to N_2 (or alternate substrate) reduction since, in the absence of these, the enzyme from either *A. vinelandii* or *C. pasteurianum* catalyzes an ATP-dependent H_2 evolution (Bulen *et al.*, 1965; Burns and Bulen, 1965; Burns, 1965). Both the Fe–Mo–protein (I) and the Fe–protein (II) components of the nitrogenase complex from *A. vinelandii* are required for ATP hydrolysis and associated reactions (Bulen and LeComte, 1966). These proteins appear similar in terms of their requirements and general properties to those obtained from *C. pasteurianum* (molybdoferredoxin and azoferredoxin) (Mortenson *et al.*, 1967; Kennedy *et al.*, 1968) and from *A. chroococcum* (Kelly, 1969).

A lack of agreement exists about the quantity of ATP required in the nitrogenase-catalyzed reactions. Because of the nature of the electron distribution between H_2 evolution and substrate reduction, ATP utilization is conveniently referred to as the ATP:$2e^-$ ratio. With *A. vinelandii* preparations of either the nitrogenase complex or recombined Fe–Mo–protein and Fe–protein components, ATP:$2e^-$ ratios of *ca.* 5 were observed for both ATP-dependent H_2 evolution and N_2 reduction (Bulen and LeComte, 1966). With dialyzed, heated extracts or a resuspended pellet preparation obtained by prolonged high-speed centrifugation, ATP:$2e^-$ ratios of 2 were reported for H_2 evolution (Hardy and Knight, 1966).

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TABLE I: ATP Utilization as a Function of Electron Distribution between H_2 Evolution and N_2 Reduction.[a]

N_2 in Ar (%)	H_2 Evolution (μmoles)	N_2 Reduction (μmoles)	H_2:N_2	$2e^-$ (μmoles)	P_i (μmoles)	ATP:$2e^-$
100	3.50	2.40	1.46	10.7	52.9	5.0 ± 0.1
75	3.64	2.22	1.64	10.3	51.2	5.0 ± 0.1
50	4.23	2.09	2.03	10.5	52.3	5.0 ± 0.2
25	4.85	1.76	2.76	10.2	49.7	4.9 ± 0.3
0	9.47			9.5	47.5	5.0 ± 0.2

[a] Assay reaction mixtures contained in 2 ml: 100 μmoles of *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid, 10 μmoles of ATP, 100 μmoles of creatine phosphate, 40 μmoles of $Na_2S_2O_4$ (all of the preceding were adjusted to pH 7.25), 10 μmoles of $MgCl_2$, 0.25 mg of creatine kinase, and P-150 nitrogenase. N_2 pressure as indicated with argon added to 1 atm. Incubation 30 min at 30° with shaking (120 oscillations/min). The data for each pN_2 represent the mean value of six reactions with two controls (calculated standard deviation indicated for each ATP:2e value).

From data obtained with Fe–Mo–protein and Fe–protein fractions from *A. chroococcum*, ATP:$2e^-$ values from 0.7 to 18 were calculated (Kelly, 1969).

With enzyme preparations from *C. pasteurianum*, ATP:$2e^-$ values of 4 for H_2 evolution or N_2 reduction were calculated from data obtained with recombined fractions (D_1 and D_2) (Mortenson, 1966) and subsequently with purified Fe–Mo–protein and Fe–protein components (Kennedy *et al.*, 1968). In the latter experiments an extract of ammonia-grown cells was no longer required and substrate levels of ATP replaced the previously used acetyl phosphate generating system. With an ethanol or acetone precipitated fraction from *C. pasteurianum*, ATP:$2e^-$ ratios from 4.0 to 4.6 that were pH dependent were reported by Winter and Burris (1968).

The significance of the ATP:$2e^-$ ratio in the reactions catalyzed by nitrogenase cannot at present be fully evaluated but neither can it be ignored in considerations of the reaction mechanism. Since the ratio of 2 was reported by Hardy and Knight (1966) using *A. vinelandii* extracts, we have periodically reexamined preparations of the nitrogenase complex from this organism for the efficiency of ATP utilization. The intact complex was used in these experiments in an attempt to minimize the possibility of ATP hydrolysis resulting from unknown but possible "uncoupling" phenomena. Special emphasis was placed on the use of control reactions corresponding to each variable examined. The ATP:$2e^-$ ratio was examined in H_2 evolution and N_2 or C_2H_2 reduction reactions. The effects on this ratio of the purity of the nitrogenase, the partial pressure of N_2 or C_2H_2 in Ar (which varies the distribution of electrons between H_2 evolution and substrate reduction), H_2 inhibition, pH, and temperature are the subject of this report.

Experimental Procedures

ATP was purchased from the Sigma Chemical Co., $Na_2S_2O_4$ from the Mallinckrodt Chemical Works, and sodium cacodylate from the Fisher Scientific Co. Creatine phosphate was synthesized in our laboratory by Dr. James Corbin. Creatine kinase was purchased from the Nutritional Biochemical Corp. Samples of *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid, *N*,*N*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid, and piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) were the generous gift of Dr. Norman E. Good. *N*-Tris(hydroxymethyl)-methyl-2-aminoethanesulfonic acid was subsequently synthesized as described by Good *et al.* (1966).

Prepurified N_2 was purchased from the Matheson Co. and H_2, argon, and C_2H_2 were obtained from the Air Reduction Co. The premixed N_2–Ar and N_2–H_2 mixtures were supplied by the Ohio Chemical and Surgical Equipment Co., Cleveland, Ohio.

Nitrogenase enzyme preparations were isolated from *A. vinelandii*. The polyacrylamide gel fraction, P-150, was prepared as described by Bulen and LeComte (1966) by substituting Bio-Gel P-150 for the P-200 column used previously. Additional purification was achieved either by $MgCl_2$ precipitation (twice) of the P-150 fraction or later by separation of the nitrogenase complex from the protamine sulfate fraction (see previous reference) by $MgCl_2$ precipitation (twice) followed by chromatography on Sephadex G-150. The preparations of purified nitrogenase complex were routinely examined in the ultracentrifuge and the purity was estimated by the symmetry of the observed refractive index gradients and the area under the curves. Normally samples containing *ca.* 10 mg of protein/ml were examined and contaminants, if detected, accounted for no more than 5% of the total area under the curves. The refractive index gradient associated with purified nitrogenase complex gave a calculated $s^0_{20,w}$ value of 11.9 S. Details of the isolation procedures yielding purified nitrogenase complex will be the subject of a separate communication. The small amounts of contaminating material, when detectable, did not effect the P_i content of control reactions or the ATP:$2e^-$ ratios. P-150 preparations were stored for short periods at room temperatures under Ar; purified nitrogenase complex was stored at 0° under Ar or H_2 where it lost little activity for periods up to 10 days. Protein was determined with the Biuret reagent of Gornall *et al.* (1949).

N_2 reduction and H_2 evolution were measured essentially as described previously (Bulen and LeComte, 1966; Bulen *et al.*, 1965). C_2H_2 reduction was followed by assaying C_2H_4 formation with a Hewlett–Packard Model 700 dual-flame gas chromatograph equipped with an alumina column (Koch

TABLE II: ATP Utilization in a H_2-Inhibited System.[a]

N_2 in H_2 (%)	H_2 Evolution (μmoles)	N_2 Reduction (μmoles)	H_2:N_2	$2e^-$ (μmoles)	P_i (μmoles)	ATP:$2e^-$
100	4.86	3.07	1.58	14.1	69.2	4.9 ± 0.2
75	6.65	1.97	3.38	12.6	62.9	5.0 ± 0.2
50	8.56	1.44	5.94	12.9	61.7	4.8 ± 0.2
25	10.08	0.58	17.68	11.8	59.4	5.0 ± 0.2
0	11.83			11.8	59.8	5.1 ± 0.4

[a] Reaction mixtures and conditions as given in Table I except for the presence of H_2 as indicated. The data for each pN_2 represents the mean value of six reactions with two controls (calculated standard deviation indicated for each ATP:2e value).

and Evans, 1966). H_2 evolved during C_2H_2 reduction was estimated with a Finnigan 1015 mass spectrometer equipped with a special gas inlet system that provided reproducible sample volumes and pressures.

Since ATP and ADP above 5 mM are inhibitory (Bulen *et al.*, 1965), a creatine phosphate–creatine kinase-generating system was used in all reactions. The P_i released was measured as previously described (Bulen and LeComte, 1966) by an adaptation of the method of Furchgott and de Gubareff (1956) for the determination of P_i in the presence of creatine phosphate. Control reactions lacking $Na_2S_2O_4$ were run with each set of complete reactions and all P_i values reported are corrected values. Reported values were also corrected for any reaction time occurring between the measurement of H_2 evolution or removal of samples for reduced product determination and the removal of samples for P_i analysis.

Results

Effect of the Partial Pressure of N_2. The distribution of electrons between ATP-dependent H_2 evolution and N_2 reduction was altered by controlling the partial pressure of N_2 over the reaction mixture. The data in Table I show a wide electron distribution between H_2 evolution and N_2 reduction but a constant $2e^-$ total. There was no significant variation in the total P_i formed giving constant ATP:$2e^-$ ratios approaching 5. Additional data from experiments at lower pN_2 values subjected to Lineweaver–Burk plots gave a K_m value of 0.18 atm. This value agrees with those observed with extracts of *A. vinelandii* by Hardy and Knight (1967) and Strandberg and Wilson (1967).

It is noteworthy that although the greatest decrease in H_2 evolution had occurred at 25% N_2, H_2 evolution is not completely inhibited at 100% N_2. In separate experiments, neither N_2 nor P_i varied significantly in identical reactions run under 1 *vs.* 6 atm of N_2. This indicates the impossibility of saturating the enzyme with N_2 to the extent that all H_2 evolution is prevented and raises the interesting possibility that the formation and displacement of H_2 at a binding site may be part of the mechanism of N_2 binding.

Hydrogen Inhibition. H_2 is a competitive inhibitor of N_2 reduction (Strandberg and Wilson, 1967; Lockshin and Burris, 1965) but does not inhibit ATP-dependent H_2 evolution (Burns and Bulen, 1965). Varying the pN_2 in H_2 thus provided an opportunity to examine ATP utilization in an inhibited system. The data in Table II show, as expected, a more rapid decrease in N_2 reduction with decreasing pN_2 than was observed with Ar as the diluent gas. The concomitant increase in H_2 evolution, however, provided a constant $2e^-$ level accompanied by a constant ATP hydrolysis. ATP:$2e^-$ ratio values again centered around 5. H_2 inhibition was competitive with an average K_i value of 0.19 atm.

pH. An adequate buffer system was required to establish the pH optimum for the azotobacter nitrogenase prior to examining the effect of pH on the utilization of ATP. Phosphate inhibits the enzyme (Bulen *et al.*, 1965) and the potassium cacodylate buffer used previously, though less inhibitory, produces hazardous volatile arsines (Brookes and Jacobs, 1958) in the presence of reducing agents like $Na_2S_2O_4$. Figure 1 shows the effect of increasing concentrations of cacodylate and four of the amphoteric buffers described by Good *et al.* (1966). *N*-Tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid, *N,N*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid, and *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid inhibited even less than cacodylate but piperazine-*N,N'*-

TABLE III: ATP Utilization in H_2 Evolution as a Function of pH.[a]

Expt	pH	H_2 (μmoles)	P_i (μmoles)	ATP:$2e^-$
1	7.0	7.28	36.5	5.0
2	7.25	7.89	38.0	4.8
3	7.4	7.90	39.7	5.0
4	7.0	8.62	42.8	5.0
5	7.25	8.33	40.8	5.0
6	7.5	8.49	39.6	4.7
7	6.0	8.05	39.7	4.9
8	7.25	10.82	53.2	4.9
9	8.0	9.34	46.8	4.8

[a] Reaction mixtures and conditions as given in Table I except for buffers; expt 1–3, 50 mM cacodylate; 4–9, 50 mM *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid; pH of reagents and buffers adjusted to indicated values; atm Ar; enzymes: expt 1–6, P-150; 7–9, purified nitrogenase complex. Each reported value represents three reactions and a control run simultaneously.

TABLE IV: ATP:2e⁻ Ratios with Purified Purified Nitrogenase Complex.[a]

Expt	Gas	H_2 Evolution (μmoles)	N_2 Reduction (μmoles)	H_2:N_2	2e⁻ (μmoles)	P_i (μmoles)	ATP:2e⁻
1	N_2	3.93	2.23	1.76	10.62	51.8	4.9
2	H_2	11.25			11.25	55.7	5.0
3	Ar	10.82			10.82	53.2	4.9
4	H_2	11.72			11.72	59.5	5.1
5	Ar	11.38			11.38	55.9	4.9

[a] Reaction mixtures and conditions as given in Table I except for enzyme and incubation period; enzyme: purified nitrogenase complex; expt 1–3 (1.08 mg); expt 4–5 (1.8 mg); incubation time: expt 1–3, 30 min, expt 4–5, 20 min. Each value represents three complete reactions with one control (expt 1–3) or six complete reactions with two controls.

bis(2-ethanesulfonic acid) was quite inhibitory. *N*-Tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (pK_a = 7.3 at 30°) was selected for routine use in the assay reactions. Azotobacter nitrogenase was active over a broad pH range with an optimum at about 7.25 (Figure 2). Reaction mixtures containing 50 mM *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid at pH 7.25 showed no significant pH changes when monitored over a 30-min reaction period.

In the pH range 6–8, the ATP:2e⁻ values calculated from H_2 evolution reactions were not significantly changed to establish a round number value other than 5 (Table III). Purification of the enzyme complex beyond the P-150 stage had no effect on the observed ratios. Control reactions without $Na_2S_2O_4$ gave slightly higher P_i values at the lower pH's *e.g.*, values at pH 6 averaged 6.4 μmoles while those at pH 7.25 and 8.0 averaged 4.1 and 3.5 μmoles, respectively. This variation is believed due in part to creatine phosphate breakdown.

Enzyme Purity. With enzyme preparations containing detectable contaminants, ATP hydrolysis resulting from ATPase activity of the contaminants could conceivably alter the observed ratios. ATP:2e⁻ ratios calculated from H_2 evolution or N_2 reduction data obtained with purified nitrogenase complex prepared as described in Experimental Procedures are shown in Table IV. Two nitrogenase (purified nitrogenase complex) preparations were used in these experiments. Values for the ATP:2e⁻ ratios all approach 5.

Reductant Independent ATP Hydrolysis. The P_i released in control reactions lacking $Na_2S_2O_4$ was generally observed to be about 15% of the total in a 30-min reaction at 30° and appeared even in reactions in which enzyme preparations of the highest purity were used. In order to examine the validity of subtracting from the total the amounts of P_i formed in control reactions lacking $Na_2S_2O_4$, we examined the P_i value of reactions lacking reductant, nitrogenase, and ATP, but retaining the ATP-generating system. Results typical of those given with the levels of nitrogenase (up to *ca.* 0.6 mg of protein/ml) normally used in assays of H_2

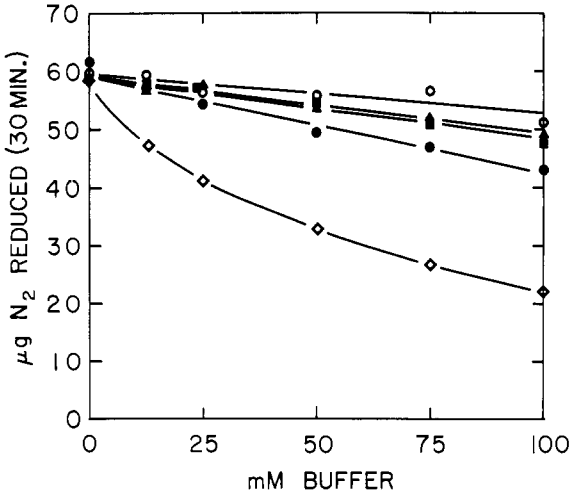

FIGURE 1: Effect of buffer concentration on N_2 reduction activity. Assay reaction mixtures and conditions as given in Table I except the following buffers were used at the concentrations indicated: (-○—○-) *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid; (-▲—▲-) *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; (-■—■-) *N*,*N*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid; (-◇—◇-) piperazine-*N*,*N'*-bis(2-ehanesulfonic acid); (-●—●-) potassium cacodylate. Atm: N_2. Enzyme: P-150. Control reactions lacked creatine phosphate, creatine kinase, and ATP.

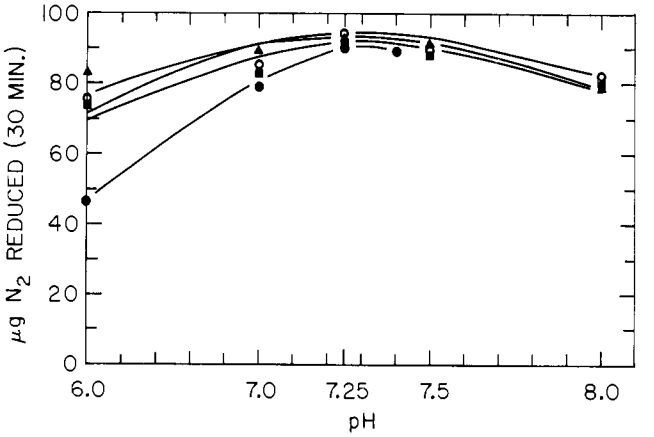

FIGURE 2: pH optimum for N_2 reduction. Assay reaction mixtures and conditions as in Table I except the pH was varied as indicated and the following buffers (50 mM) were used: (-○—○-) *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid; (-▲—▲-) *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; (-■—■-) *N*,*N*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid; (-●—●-) potassium cacodylate. Atm: N_2. Enzyme: P-150. Control reactions lacked creatine phosphate, creatine kinase, and ATP.

TABLE V: P_i Formed from Reagents and Nonreductant ATP Hydrolysis.[a]

Reaction	P_i (μmoles)	P_i (%)
Complete	58.4	100
Minus $Na_2S_2O_4$	8.1	13.8
Minus enzyme, $Na_2S_2O_4$	5.3	9.1
Minus ATP	5.3	9.1

[a] Reaction mixture and conditions as given in Table I except as indicated. Each value represents the average of three reactions; enzyme: purified nitrogenase complex (1.05 mg).

evolution, N_2, or acetylene reduction are presented in Table V. The control P_i values thus consist of P_i originating from the generating system and P_i originating from an enzymic hydrolysis of ATP that is independent of reductant. Other experiments in our laboratory have shown that the nitrogenase-catalyzed hydrolysis of ATP in the absence of reductant increases with ATP concentration (R. Silverstein, unpublished data). Together these results establish the ability of nitrogenase to catalyze a reductant independent hydrolysis of ATP. The magnitude of this activity at the enzyme concentrations used here for determining ATP:$2e^-$ values is, however, barely within experimental error.

Acetylene Reduction. With the discovery of the reduction of acetylene to ethylene (Dilworth, 1966; Schöllhorn and Burris, 1967), it seemed advisable to check the effect of a second reducible substrate on the utilization of ATP. The data obtained (Table VI) with a saturating level of C_2H_2 showed nearly 90% of electron pairs used for C_2H_2 reduction with about 10% going to H_2 evolution. With a limiting level of C_2H_2, the portion of electrons going to hydrogen evolution increases. The ATP:$2e^-$ ratios, though showing greater variation resulting from the measurement of H_2 in the mass spectrometer, still approach 5.

Variation of ATP:$2e^-$ Ratio with Temperature. If the ATP:$2e^-$ ratio represented classical, single-reaction stoichiometry, it should remain constant even though the rate of reaction was increased by an increase in temperature. To test this concept, the ATP-dependent H_2 evolution reaction was examined over the range 20–40° at pH 7.25. Enzyme concentrations were selected such that the H_2 evolved was not less than 5 μmoles and the total P_i released was not less than 30 μmoles. Each series of reactions was accompanied by control reactions lacking $Na_2S_2O_4$ (range 7–13 μmoles of P_i) and each point plotted in Figure 3 was calculated from the data of 6–12 complete reactions. These data show a definite increase in the ATP:$2e^-$ ratio with temperature over the range tested.

Discussion

The experiments described here represent an effort to extend our previous observations about ATP utilization with *A. vinelandii* nitrogenase and an attempt to contribute to an understanding of the apparent lack of agreement among the ATP:$2e^-$ values observed in different laboratories.

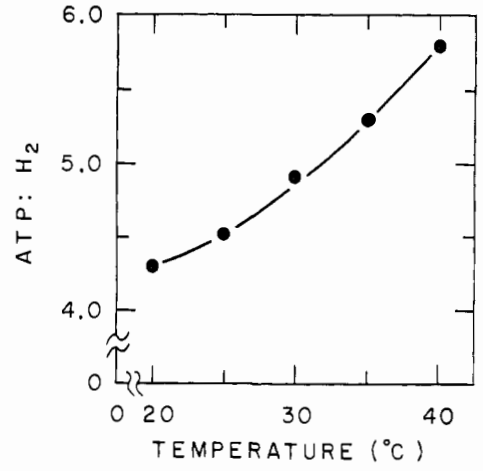

FIGURE 3: Temperature dependence of the ATP:$2e^-$ ratio. Assay reaction mixtures and conditions as in Table I. Atm: Ar. enzyme: purified nitrogenase complex.

The conditions selected for ATP utilization measurements merit a brief discussion which may help the reader to evaluate our results and those of others. Enzyme purification to the polyacrylamide gel stage (P-150) was adequate to remove contaminating ATPase activity to a level easily corrected for in the controls. Hydrogenase, even if present, would not reduce the ATP:$2e^-$ values because, in contrast to the clostridial enzyme, hydrogenase from *Azotobacter* sp. does not evolve H_2 even in the presence of $Na_2S_2O_4$. The use of the nitrogenase complex was selected to reduce the chances of an "uncoupling phenomena" (if such exists) and to eliminate the need for recombining components in the proper proportions. The ATP-generating system was selected because ATP:$2e^-$ ratios determined at substrate levels of ATP involve a system inhibited both by the substrate and products (ADP and P_i) and require low levels of activity or short reaction times both of which contribute to the errors associated with product determinations. In addition, ATP concentrations above 2 mM interfere with the color development in the molybdenum blue method (Stanton, 1968) and, if this is presumed to represent an inhibited formation of the polymolybdophosphoric acid which is subsequently reduced (Crouch and Malmstadt, 1967), ADP may have the same effect. The ATP interference is particularly pronounced

TABLE VI: Acetylene Reduction.[a]

C_2H_2 (torr)	H_2	C_2H_4	$2e^-$	P_i	ATP: $2e^-$
10	6.0	7.20	13.2	62.9	4.8
75	1.0	11.7	12.7	63.0	4.9

[a] Reaction mixture and conditions as given in Table I. Argon added to give total pressure of 1 atm; enyzme: purified nitrogenase complex. Values represents three complete reactions each for C_2H_2 reduction and H_2 evolution. Columns 2–5 are given in micromoles.

if the color intensity is recorded after 2 min. ATP utilization was assayed as P_i released rather than as creatine formed from the generating system to ensure the inclusion of any P_i hydrolyzed from ATP that was not regenerated and to minimize the uncertainty about creatinine formation. Finally, each manifold of three complete reactions was accompanied by a control lacking $Na_2S_2O_4$ at each pH, temperature, gas atmosphere, etc., examined to correct for any nonenzymic P_i formation, contaminating ATPase activity if any, and reductant independent ATP hydrolysis catalyzed by the nitrogenase. P_i levels in control reactions varied slightly among nitrogenase preparations, increased somewhat with the age of the preparations, and increased both at the lower pH values and at the higher temperatures.

Both this and our previous work establish an ATP:$2e^-$ ratio of *ca.* 5 at 30°. We can only conclude that the substantially lower values for H_2 evolution or acetylene reduction reported by others using azotobacter nitrogenase at 30° resulted from some experimental error.

Discrepancies between values observed with the clostridal and the azotobacter systems are probably most resolvable in terms of reaction temperature. The most pure, hydrogenase free, clostridial preparations used to date are probably those of Kennedy *et al.* (1968) who observed an average ATP:$2e^-$ value of 4.15. These reactions were conducted at 22°. The data in Figure 3 show that the azotobacter preparations gave an average value of 4.3 at 20°. A discrepancy still exists about the dependence of the ATP:$2e^-$ ratio on pH since we did not observe reproducible effects (Figure 3) of the magnitude reported by Winter and Burris (1968). There is, of course, at present no experimentally substantiated reason to believe that the two systems must function exactly the same. They do, in fact, exhibit different pH optima though both are broad.

Although we report our ATP:$2e^-$ ratio values as approaching 5 at 30°, we question the necessity for or validity of a whole number value for this ratio and even the application of the concept of reaction stoichiometry in this case. The dependence of the ratio upon temperature suggests at least two reactions with different activation energies, one leading to H_2 evolution or substrate reduction accompanied by ATP hydrolysis and another providing only ATP hydrolysis. This could result from the reaction of a common enzyme–ATP complex in two ways, both leading to ATP hydrolysis but only one providing reducing electrons. The concept of two reactions with different activation energies is inconsistent with the claim by Burns (1969) of identical activation energy values for N_2 reduction, H_2 evolution, and P_i release (except under the unlikely circumstance where both ATP-hydrolyzing processes had the same energy of activation). For the latter to be true, an Arrhenius plot based on P_i release should exactly parallel those for H_2 evolution and N_2 reduction. For temperatures above 20° the data presented by Burns for P_i formation do not convincingly substantiate this claim. Since the P_i released would represent the sum of both proposed reactions, small differences in the slopes of Arrhenius plots could become quite significant. This point would merit additional investigation.

Acknowledgments

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