Shah, V. K., Davis, L. C., & Brill, W. J. (1972) *Biochim. Biophys. Acta 256*, 498-511.
Shah, V. K., Davis, L. C., Gordon, J. K., Orme-Johnson, W. H., & Brill, W. J. (1973) *Biochim. Biophys. Acta 292*, 246-270.
Silverstein, R., & Bulen, W. A. (1970) *Biochemistry 9*, 3809-3815.
Smith, B. E. (1977) *J. Less-Common Metals 54*, 465-475.
Thorneley, R. N. F., Eady, R. R., & Yates, M. G. (1975) *Biochim. Biophys. Acta 403*, 269-284.
Vandercasteele, J.-P., & Burris, R. H. (1970) *J. Bacteriol. 101*, 794-801.
Watt, G. D. (1980) in *Molybdenum Chemistry of Biological Significance* (Newton, W. E., & Otsuka, S., Eds.) pp 3-21, Plenum Press, New York.
Watt, G. D., & Burns, A. (1977) *Biochemistry 16*, 264-270.
Wilson, P. W., & Umbreit, W. W. (1937) *Arch. Mikrobiol. 8*, 440-457.
Winter, H. C., & Burris, R. J. (1976) *Annu. Rev. Biochem. 45*, 409-426.

Nitrogenase Reactivity: Insight into the Nitrogen-Fixing Process through Hydrogen-Inhibition and HD-Forming Reactions†

Barbara K. Burgess, Scot Wherland,‡ William E. Newton,* and Edward I. Stiefel

ABSTRACT: The dihydrogen reactions of nitrogenase are H_2 evolution, H_2 inhibition of N_2 reduction, and HD production from H_2/D_2O or D_2/H_2O. The relationships among these dihydrogen reactions are studied to gain insight into the mechanism of N_2 reduction. Detailed studies have probed (1) the formation of HD by nitrogenase as a function of partial pressures of N_2, D_2, and CO, (2) the formation of TOH from T_2 under N_2-fixing conditions, and (3) the reduction of hydrazine by nitrogenase. Experiments under T_2 demonstrate that negligible tritium is incorporated into water compared to the HD produced under similar conditions. Studies of total electron flow, in the presence or absence of D_2, establish a requirement of 1 mol of electrons/mol of HD formed. These findings show definitively that HD formation is *not* due to a simple H_2O/D_2 exchange mechanism. Kinetic analysis shows that HD is produced by two separate processes. In the minor process, the HD formed is proportional to the H_2 evolved, electron requiring, and partially inhibited by 1% CO. In the major process, HD formation is dependent on N_2 pressure, electron requiring, and completely inhibited by CO. A mechanism is proposed whereby HD from the N_2-dependent process is formed from a bound, reduced dinitrogen intermediate. This mechanism is supported by studies using hydrazine as a substrate for nitrogenase and leads to the conclusion that H_2 inhibition of nitrogen fixation and N_2-dependent HD formation are manifestations of the same molecular process.

The dihydrogen reactions of nitrogenase have been extensively studied in vitro (Jackson et al., 1968; Bulen, 1976; Stiefel et al., 1977; Newton et al., 1977) and in vivo [e.g., Evans et al. (1980)]. In the absence of other reducible substrates, all of the reductant consumed by nitrogenase is used to reduce protons to H_2 in the ATP[1]-dependent H_2 evolution reaction (Bulen et al., 1965). When N_2 is added as a reducible substrate, an extrapolated maximum of 75% of the electrons reduce N_2 while the remainder reduce protons (Hadfield & Bulen, 1969; Rivera-Ortiz & Burris, 1975). Dihydrogen is not only a product of nitrogenase turnover but is also an inhibitor of N_2 reduction (Wilson & Umbreit, 1937). H_2 (and, by implication, D_2) inhibition is specific for N_2 reduction and does not affect either the reduction of other nitrogenase substrates or its own evolution (Hwang et al., 1973). The apparent competitive nature of this inhibition was first demonstrated in red clover plants (Wilson & Umbreit, 1937) and later in other organisms including *Azotobacter vinelandii* (*Av*) (Strandberg & Wilson, 1967; Hadfield & Bulen, 1969; Hwang et al., 1973).

Hoch et al. (1960) showed that both H_2 evolution and HD formation occurred in soybean nodules under a D_2/N_2 atmosphere and that the latter was stimulated by N_2 and inhibited by either CO (an inhibitor of N_2 reduction) or N_2O (an alternative substrate). They suggested that HD formation occurred by a reversible exchange of D_2 with an enzyme-bound diazene intermediate (Hoch et al., 1960). However, later attempts to trap and so identify diazene as an intermediate in N_2 reduction were unsuccessful (Burris et al., 1965). Other workers confirmed these results with both the soybean nodule system (Bergerson, 1963; Turner & Bergerson, 1969) and cell-free preparations from the free-living, N_2-fixing microorganism *A. vinelandii* (Jackson et al., 1968). These studies also showed that HD formation required ATP and reductant and supported speculation that HD was formed by a reversible exchange of D_2 with an enzyme-bound diazene-, hydrazine-, and/or amine-level intermediate. Hwang et al. (1973) reported that the enhancement of HD formation by N_2 was more pronounced for *Av* nitrogenase than for the *Clostridium pasteurianum* system.

Recognizing the potential for obtaining information concerning the mechanism of N_2 reduction from quantitation of this phenomenon, Bulen (1976), using the purified *Av* nitrogenase complex (Hadfield & Bulen, 1969; Bulen & Le-

† Contribution No. 733 from the Charles F. Kettering Research Laboratory, Yellow Springs, Ohio 45387. *Received July 29, 1980; revised manuscript received April 2, 1981.* This study was supported in part by Project No. 79-00359 from SEA/CGO of the U.S. Department of Agriculture. S.W. was supported by a National Science Foundation National Needs Postdoctoral Fellowship.

‡ Present address: Department of Chemistry, Washington State University, Pullman, WA 99163.

[1] Abbreviations used: ATP, adenosine 5′-triphosphate; $NaDodSO_4$, sodium dodecyl sulfate, cpm, counts per minute; dppe, 1,2-bis(diphenylphosphino)ethane.

0006-2960/81/0420-5140$01.25/0

Comte, 1972), reported that HD formation was electron requiring and, thus, could not be formed by reversible exchange with the solvent (Newton et al., 1976, 1977). These studies have been confirmed and extended recently in our laboratory by using both the *Av* nitrogenase complex (Newton et al., 1977; Stiefel et al., 1977) and the purified component proteins (Stiefel et al., 1980; Burgess et al., 1980a; Wherland et al., 1981) and have led to a mechanistic interpretation that differs significantly from those postulated previously (Hoch et al., 1960; Jackson et al., 1968). We have suggested that H_2 inhibition of N_2 fixation and HD formation under D_2 and N_2 are manifestations of the same molecular process, which involves a bound diazene-level intermediate formed during the reduction of N_2 to ammonia. Further experiments probing HD formation, H_2 inhibition of N_2 reduction, hydrazine reduction, and tritium exchange using highly purified component proteins of nitrogenase are reported here.

Materials and Methods

Nitrogenase. The two component proteins of *Av* nitrogenase, the molybdenum–iron protein (*Av*1) and the iron protein (*Av*2), were purified by a method developed in our laboratory (Burgess et al., 1980c). Both components appeared homogeneous by $NaDodSO_4$ gel electrophoresis and were shown to be devoid of uptake hydrogenase activity by the tritium exchange assay described below. The specific activity of *Av*2 was 1950–2100 nmol of H_2 evolved min^{-1} (mg of *Av*2)$^{-1}$ and of *Av*1 was 2800–3000 nmol of H_2 evolved min^{-1} (mg of *Av*1)$^{-1}$. These activities were observed at the molar component ratio *Av*2/*Av*1 of ~1.0 for *Av*2 and 40 for *Av*1 on the basis of a molecular weight of 64 000 for *Av*2 and 230 000 for *Av*1.

Nitrogenase Assay. All assays, except the $Na_2S_2O_2$ utilization experiments (see below), were performed as described in Wherland et al. (1981) and contained a total of 1 mg of protein per 1 mL reaction. Products were analyzed and data converted to nanomodes of product per minute per milligram of total protein (Wherland et al., 1981). We emphasize that in this paper rates are expressed per milligram of total protein (milligrams of *Av*1 plus milligrams of *Av*2) and must be converted for comparison with specific activity. Unless otherwise indicated, all experiments were performed at the molar ratio *Av*2/*Av*1 of 5.

Calculations. Calculations were performed as described in the preceding paper (Wherland et al., 1981). The line drawn in Figure 4 is a least-squares fit to eq 1 of all the individual data points. The K_m values reported are calculated from a least-squares fit of all data points.

$Na_2S_2O_4$ Utilization. $Na_2S_2O_4$ utilization was measured by using a polarographic technique (Watt & Burns, 1977). The reaction mixture contained the reactants in the appropriate proportions in 2.5 mL (Wherland et al., 1981). The polarographic cell was flushed with the appropriate gas mixture throughout the reaction.

Hydrazine Reduction. The concentrations shown in Table SII (see paragraph at end of paper regarding supplementary material) and in Figure 3 are of hydrazine neutralized to pH 7.4. At pH 7.4, only 37% of the hydrazine added is in the neutral N_2H_4 form (Bulen, 1976).

Tritium Exchange. The gas mixture used contained 48.4% H_2/40.5% N_2/balance Ar enriched with 20.5 mCi/L of tritium (Matheson). Reactions were run as above except that assay vials were degassed after quenching to remove H_2 (T_2) dissolved in the liquid phase. Reaction mixtures (0.75 mL) were added to 10 mL of Aquasol, and the vials were counted for 30 min in a Packard Model 2002 liquid scintillation counter. Controls in triplicate contained the reaction mixture alone, the reaction mixture plus 1 mg of *Av*1, and the complete system without $Na_2S_2O_4$. These nine vials gave 23.1 ± 5.5 cpm, corresponding to 2 nmol of H^+ (labeled with T^+) min^{-1} (mg of protein)$^{-1}$ incorporated. The average of five experimental vials was 87 ± 10.2 cpm, corresponding to 5.7 nmol of H^+ (labeled with T^+) incorporated after correcting for the background. Quenching was determined by comparing experimental vials to Packard quenched standards.

Table I: Dithionite Utilization Studies

gas	μmol of e^- min^{-1} (mg of protein)$^{-1}$
100% Ar	1.20 ± 0.06
100% N_2	1.04 ± 0.10
40% N_2/60% Ar	1.04 ± 0.10
40% N_2/10% Ar/50% H_2	1.05 ± 0.12

Gases. All gases were of the highest purity available and were analyzed by Matheson. Gases used were as follows: 50% D_2/50% Ar (analyzed at <0.0005% N_2); 50% D_2/0.481% N_2/Ar balance; 50% D_2/0.944% N_2/Ar balance; 50% D_2 1.8% N_2/Ar balance; 50% D_2/3.61% N_2/Ar balance; 50% D_2/7.66% N_2/Ar balance; 50% D_2/9.99% N_2/Ar balance; 50% D_2/19.59% N_2/Ar balance; 50% D_2/39.9% N_2/Ar balance.

Chemicals. All chemicals used were highest grade available from the Sigma Chemical Co. except creatine phosphate, which was synthesized according to published methods (Anatol, 1961).

Results

H_2 Inhibition of N_2 Reduction. Under an atmosphere of 40% N_2/60% Ar and at a molar ratio *Av*2/*Av*1 of 5, nitrogenase catalyzes the production of 156 nmol of NH_3 min^{-1} (mg of protein)$^{-1}$. Under 50%H_2/40%N_2/10% Ar and at the same ratio, ammonia production decreases to 77 ± 1 nmol of NH_3 min^{-1} (mg of protein)$^{-1}$, which represents a 50% inhibition of N_2 reduction by 50% H_2. With 50% D_2/40% N_2/10% Ar and eight ratios of *Av*2/*Av*1 ranging from 0.5 to 18.0, the average inhibition of N_2 reduction by 50% D_2 is 49 ± 5% (Wherland et al., 1981). These results are consistent with previous results (Wilson & Umbreit, 1937) and demonstrate that H_2 is a significant inhibitor of N_2 reduction.

HD Formation Catalyzed by Nitrogenase. Under an atmosphere of 50%D_2/40% N_2/10% Ar, nitrogenase (molar ratio *Av*2/*Av*1 of 5) catalyzes the production of 360 ± 51 nmol of H_2 min^{-1} (mg of protein)$^{-1}$, 266 ± 24 nmol of HD min^{-1} (mg of protein)$^{-1}$, and 89 ± 11 nmol of NH_3 min^{-1} (mg of protein)$^{-1}$. When 1% CO, a potent inhibitor of all nitrogenase-catalyzed reactions except H_2 evolution (Bulen & LeComte, 1966), is included in the above gas mixture, H_2 evolution increases to 670 ± 50 nmol min^{-1} (mg of protein)$^{-1}$, HD formation decreases by 89% to 30 ± 2 nmol min^{-1} (mg of protein)$^{-1}$, and no NH_3 is detected. No HD formation occurs with either *Av*2 or *Av*1 alone or with both proteins present if either MgATP or $Na_2S_2O_4$ is absent.

Electron Requirement for HD Formation. Table I shows that the rate of $Na_2S_2O_4$ utilization is the *same* under N_2-fixing conditions or H_2-inhibited, N_2-fixing conditions and very similar to that under H_2-evolving conditions (Wherland et al., 1981). These results are consistent with previous studies in which nitrogenase turnover was shown to be virtually independent of the substrate being reduced (Watt & Burns, 1977). Figure 1 is a plot of total electron flow through nitrogenase under D_2-inhibited, N_2-fixing conditions as a function of the partial pressure of N_2 (p_{N_2}). When two electrons are allocated for each H_2 evolved and three for each NH_3 formed, but none for HD formation, total electron flow decreases by 25% as p_{N_2}

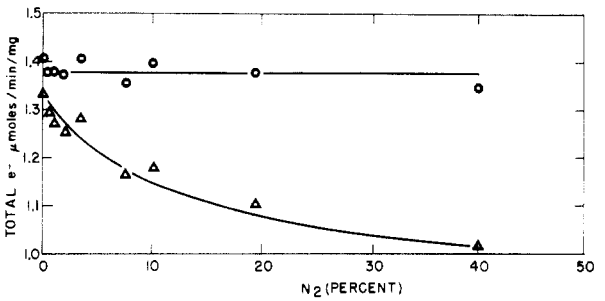


FIGURE 1: Plots of total electron flow under 50% D_2 as a function of N_2 pressure with one electron (O) and zero electrons (Δ) assigned for each HD formed. Assay conditions are described under Materials and Methods.

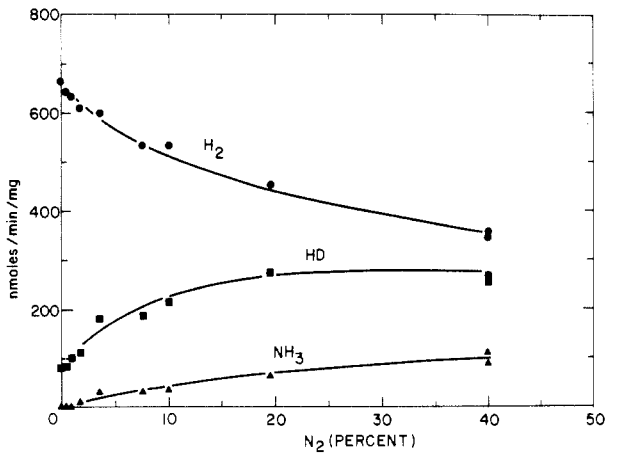


FIGURE 2: Plots of the rate of NH_3 (▲), HD (■), and H_2 (●) production under 50% D_2 as a function of N_2 pressure. Assay conditions are as described under Materials and Methods.

increases from 0% to 40%. However, if one electron is allocated for each HD formed, then the total electron flow as a function of p_{N_2} becomes consistent with the $Na_2S_2O_4$ utilization data.

Because HD formation might occur by the simple reversible exchange mechanism, nitrogenase turnover was examined

$$D_2(g) + H_2O(l) \rightleftarrows HD(g) + HOD(l)$$

under 50% H_2 (labeled with T_2)/40% N_2/10% Ar. Only 5.7 ± 1.0 nmol of H^+ (labeled with T^+) min^{-1} (mg of protein)$^{-1}$ was incorporated into the aqueous phase compared with 266 ± 24 nmol of HD min^{-1} (mg of protein)$^{-1}$ evolved under 50% D_2/40% N_2/10% Ar. Thus, the amount of HD formed is ~50 times greater than the amount of labeled H^+ incorporated into the liquid phase, a result inconsistent with a reversible exchange mechanism.

N_2 Dependence of HD Formation. Figure 2 shows the rates of H_2 evolution, ammonia production, and HD formation as functions of p_{N_2}. As p_{N_2} increases, H_2 evolution decreases while both ammonia production and HD formation increase. This figure shows a significant amount of HD in the absence of N_2. These results are consistent with those using the *Av* nitrogenase complex (Bulen, 1976), where a very small amount of N_2 contaminating the D_2/Ar mixture was suggested to act as a "catalyst" for HD production. An alternative interpretation, however, is that a second pathway for HD formation exists, which is N_2 independent. Because the effect is most apparent at low p_{N_2}, we measured HD formation as a function of p_{N_2} at seven N_2 levels below 0.1 atm (Figure 2). Under 50% D_2/50% Ar, where no N_2 is detectable, 81 ± 9 nmol of HD min^{-1} (mg of protein)$^{-1}$ is formed. When p_{N_2} is increased to 0.005 atm, 85 ± 3 nmol of HD min^{-1} (mg of protein)$^{-1}$ is

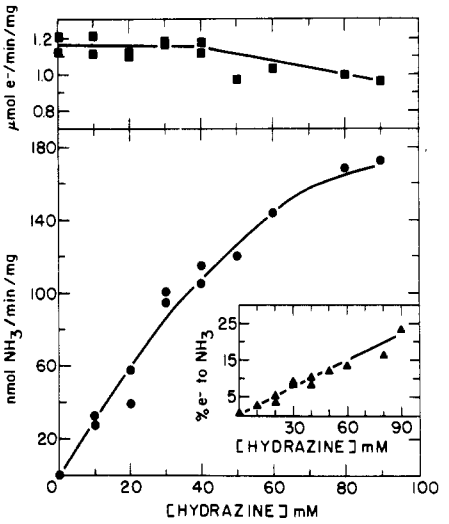


FIGURE 3: Plots of the rates of NH_3 (●) formation and total electron flow [(■) calculated as two electrons per H_2 and one electron per NH_3] as a function of hydrazine concentration. Gas phase was 100% Ar. (Inset) Plot of the percentage of total electron flow that goes to NH_3 formation as a function of hydrazine concentration (Δ). Assay conditions are as described under Materials and Methods.

formed which does not represent a significant increase over 0 atm of N_2. HD formation begins to increase at 0.01 atm of N_2 and continues to a maximum at ~0.2 atm of N_2. A double-reciprocal plot (not shown) of these HD data is linear with a calculated apparent $K_m(N_2)$ for HD formation of 0.012 ± 0.004 atm of N_2, which is consistent with previously published results (Jackson et al., 1968; Newton et al., 1977). *However, it must be stressed that this K_m is not a physically meaningful number* because it is calculated from total HD values which actually represent the sum of HD formed by two separate paths, one N_2 independent and the other N_2 dependent.

N_2-independent HD formation has been studied further as a function of the molar component ratio (Wherland et al., 1981). The percentage of total electron flow used to form HD by this pathway was *not* dependent on the *Av2*/*Av1* ratio but was constant at 9 ± 1% for 100% D_2 and 6 ± 0.5% for 50% D_2/50% Ar over the range of ratios 0.1–120 (Wherland et al., 1981). For comparison, under 50% D_2/40% N_2/10% Ar, HD formation represents up to 25% of the total electron flow. Thus, the N_2-independent HD formation pathway represents a minor, but nevertheless significant, contribution to the total HD formed.

Hydrazine Reduction by Nitrogenase. Our results confirm hydrazine as a substrate of the recombined, purified component proteins of nitrogenase and show that hydrazine is not reduced by either *Av2* or *Av1* alone or with both components present if either MgATP or $Na_2S_2O_4$ is absent. Under 100% Ar with 30 mM hydrazine present, at a molar ratio *Av2*/*Av1* of 5, nitrogenase catalyzes the formation of 82.1 ± 5.2 nmol NH_3 min^{-1} (mg of protein)$^{-1}$. When 1% CO is added to the argon atmosphere, no ammonia formation is detected. Figure 3 is a plot of the rate of ammonia formation vs. hydrazine concentration. Also shown is the total electron flow (two electrons per H_2 and one per NH_3) through nitrogenase as a function of hydrazine concentration. Although ammonia formation appears to approach saturation over the concentration range tested, at concentrations above 40 mM hydrazine, the total electron flow begins to decrease, indicating that high levels of hydrazine inhibit nitrogenase turnover. The inset of Figure 3 shows the percentage of total electron flow that goes to ammonia formation vs. hydrazine concentration. Because this

Table II: Hydrazine Reduction under H_2 and D_2

conditions	NH_3[a]	HD[a]
100% Ar + 30 mM hydrazine	82.1 ± 5.2	
100% H_2 + 30 mM hydrazine	81.7 ± 5.3	
50% D_2/50% Ar		83 ± 1.7
50% D_2/50% Ar + 30 mM hydrazine	[b]	81 ± 3.2
50% D_2/10% Ar/40% N_2	[b]	266 ± 14.0

[a] Assays were performed as described under Materials and Methods. NH_3 and HD values represent nanomoles produced per minute per milligram of total protein. [b] Not necessary for this comparison.

percentage remains linear over the concentration range studied, hydrazine appears to be a poor substrate which does not begin to saturate nitrogenase below 30 mM, and hence no meaningful K_m can be determined. Because of the involvement of H_2 and D_2 in N_2 reduction by nitrogenase, the effects of H_2 and D_2 on hydrazine reduction were investigated. Table II shows that hydrazine reduction to ammonia is not inhibited by H_2 and does not enhance HD formation under D_2.

Discussion

All previous work on HD formation by nitrogenase was performed by using crude or partially purified nitrogenase or the *Av* nitrogenase complex, which left open the possibility that all or part of the HD formed may have arisen from contaminating hydrogenase. The results we present here, using purified *Av*2 and *Av*1 which are devoid of uptake hydrogenase activity, firmly establish HD formation as a reaction which occurs during nitrogenase turnover. Our electron-balance studies (Figure 1) confirm the conclusion drawn from our earlier work with both the *Av* nitrogenase complex (Newton et al., 1977) and purified *Av*2 and *Av*1 (Stiefel et al., 1980; Burgess et al., 1980a) that one electron is required for each HD molecule formed. Although a 13% decrease in electron flow is observed under atmospheres containing 40–100% N_2 compared to those without (Table I; Wherland et al., 1981), a much larger (25%) decrease results if the electron requirement for HD is ignored (Figure 1). When one electron per HD is added, a less obvious 7% decrease in electron flow might still occur. This electron requirement for HD formation rules against the reversible exchange mechanism, i.e., $D_2 + H_2O \rightleftarrows HD + HOD$, and leads to the prediction that while HD is formed in the gas phase, HOD is not formed concomitantly in the aqueous phase (Newton et al., 1977). This prediction is verified by our experiments with tritium-labeled H_2 which demonstrate conclusively that more than 98% of the HD formed must be attributed to a process other than a reversible exchange involving the solvent. Further, since no label appears in the solvent, interactions that lead to tritium-labeled NH_3 cannot occur since this labeled NH_3 would undergo exchange of the label with the solvent and would, in any event, be detected in the solution phase.

Considerable controversy exists particularly with respect to the N_2 dependence of HD formation by nitrogenase. All published data (Hoch et al., 1960; Bergerson, 1963; Kelly, 1968; Jackson et al., 1968; Turner & Bergerson, 1969; Bulen, 1976) show some HD formed in the absence of N_2 as do our N_2-dependence experiments. As an explanation of such results, it has been suggested that very low levels of N_2 contaminating the gas mixtures being tested are responsible for catalyzing HD formation (Bulen, 1976). However, these results could also indicate two separate pathways for HD formation, a N_2-*dependent* and a N_2-*independent* pathway. In fact, detailed studies of HD formation as a function of the ratio *Av*2/*Av*1 under 100% D_2 and 50% D_2/50% Ar (Wherland et al., 1981)

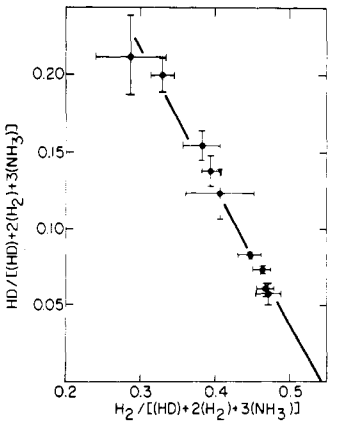


FIGURE 4: Linear least-squares fit of data in Figure 2 plotted according to eq 1. Calculated values for F and F' are $F = 0.062 \pm 0.01$ and $F' = 0.49 \pm 0.03$. $[1(HD) + 2(H_2) + 3(NH_3)]$ represents the total electron flow calculated on the basis of one electron per HD, two electrons per H_2, and three electrons per NH_3.

have firmly established the occurrence of N_2-independent HD formation. These studies showed that, although both H_2 evolution and HD formation show a marked component ratio dependence, the percentage of the total electron flow that is used to form HD is *not* dependent on the *Av*2/*Av*1 ratio but is constant at 6% and 9% for atmospheres containing 50% and 100% D_2, respectively. This observation contrasts with similar experiments under 50% D_2/40% N_2/10% Ar, where the percentage of total electron flow attributed to total HD, the majority of which is N_2 *dependent*, show a marked dependence upon the component ratio (Wherland et al., 1981).

These findings lead us to the hypothesis that, at a given level of D_2, a constant percentage of the total electron flow that would, in the absence of D_2, go to H_2 evolution is intercepted by that D_2 to form HD* (N_2-*independent* HD formation). By application of a similar argument to N_2-*dependent* HD formation, it is predicted that a constant percentage of the total electron flow, which would go to ammonia formation in the absence of D_2, is intercepted by D_2 to form HD** (N_2-*dependent* HD formation). This hypothesis, along with the other quantitative data we present here, leads directly to the formulation of eq 1.

If $HD_{total} = HD^* + HD^{**}$ and $F = HD^*/[HD^* + 2(H_2)]$, $F' = HD^{**}/[HD^{**} + 3(NH_3)]$ (where F and F' are fractions of the appropriate electron flow), and $\epsilon = HD_{total} + 2(H_2) + 3(NH_3)$ (where ϵ is the total electron flow), then

$$\frac{HD_{total}}{\epsilon} = \frac{H}{\epsilon} 2\left[\frac{2(F - F')}{1 - F}\right] + F' \qquad (1)$$

Equation 1 predicts that a plot of HD_{total}/ϵ vs. $H_{2,total}/\epsilon$ should give a straight line with an intercept of F' and a slope of $2[(F - F')/(1 - F)]$. A least-squares fit of the N_2-dependence data (Figure 2) plotted according to eq 1 is shown as Figure 4. As predicted, the data fall on a straight line, with calculated values for F of 0.062 ± 0.01 and F' of 0.49 ± 0.03. Previously published N_2-dependence data for the *Av* nitrogenase complex (Bulen, 1976), when plotted according to eq 1, also fall on the same line. These results are consistent with the formation of HD by two separate pathways. Under 50% D_2, in the minor N_2-independent pathway, D_2 redirects electrons from H_2 evolution to form HD, with 6% of the electrons being so utilized. In the major, N_2-dependent pathway under 50% D_2, D_2 redirects 50% of the electrons from ammonia formation to form HD.

Because N_2-independent HD formation redirects a constant percentage of electrons away from H_2 evolution, this HD is

predicted to form by interaction with the H_2 evolution site. Preliminary experiments demonstrate that CO, which does not inhibit H_2 evolution, only partially inhibits N_2-independent HD formation and that this HD formation shows a nonlinear dependence on D_2. A detailed understanding of the mechanism of N_2-independent HD formation must await further studies.

Early studies on the inhibition of nitrogenase-catalyzed reactions identified H_2 as a specific inhibitor of N_2 reduction (Wilson & Umbreit, 1937). Our quantitation of this inhibition shows that 50% less ammonia is formed under 50% H_2/40% N_2/10% Ar when compared to 60% Ar/40% N_2. In addition, under 50% D_2/40% N_2/10% Ar, 50% of the electrons that would have gone to ammonia appear as N_2-dependent HD. These results strongly reaffirm our earlier conclusion that N_2-*dependent* HD formation and H_2 inhibition of nitrogen fixation occur by the same stoichiometric mechanism (Newton et al., 1976, 1977; Stiefel et al., 1977). Although we would expect H_2 and D_2, as isotopes of the same element, to react similarly with nitrogenase (or its substrate complexes), it has been suggested that the inhibition of N_2 reduction by H_2 and HD formation under N_2 are reactions distinct from one another (Jackson et al., 1968). This suggestion, which was based on the requirement for N_2 in the latter reaction and the competitive reaction of N_2 and H_2 observed for some metal complexes (e.g., eq 2), produced eq 3 for the hydrogen-inhibition reaction of the enzyme (E). However, eq 3 cannot accurately represent the inhibition process because it does not conform to the observed stoichiometry.

$$Mo(N_2)_2(dppe)_2 + 2H_2 \rightleftarrows MoH_4(dppe)_2 + 2N_2 \quad (2)$$

$$E\text{–}N_2 + H_2 \rightleftarrows E\text{–}H_2 + N_2 \quad (3)$$

Our studies show that the stoichiometry of N_2-dependent HD formation (H_2 inhibition) is given by eq 4. Combining the terms [$E + N_2 + 2H^+ + 2e^-$] implies the presence of a bound diazene-level species which reacts with D_2 or H_2 during the reduction of N_2 to ammonia. Our new data are consistent with this hypothesis, which was first formulated from earlier experiments on the *Av* nitrogenase complex (Bulen, 1976; Newton et al., 1976, 1977; Stiefel et al., 1977). Thus, under H_2-inhibited, N_2-fixing conditions, one H_2 molecule reacts with a diazene-level species, e.g., $E\text{–}N_2H_2$, to form two H_2 molecules and reform N_2. Because one H_2 molecule is consumed in this reaction and two H_2 molecules are formed, the net gain is one H_2 molecule formed from the two protons and two electrons initially in the diazene-level species. Thus, we actually measure two electrons per *one* H_2 evolved, with the H_2 formed in this manner being indistinguishable from normal ATP-dependent H_2 evolution. Under D_2-inhibited conditions, D_2 reacts with the same intermediate to generate two molecules of HD and one N_2 molecule. But now, because both molecules of product (HD) are recognizably different from the reactant (D_2) and from H_2 evolved by normal ATP-dependent hydrogen evolution, we actually measure two electrons per *two* HD molecules formed. It may well be significant from an efficiency standpoint that, under a 50% $H_2(D_2)$ atmosphere, 50% of the electrons that should be used to reduce N_2 are intercepted in this fashion. Thus, a build up of H_2 in vivo could greatly decrease the efficiency of N_2 fixation.

$$E + N_2 + 2H^+ + 2e^- + D_2\ (H_2) \rightarrow E + N_2 + 2HD\ (2H_2) \quad (4)$$

This diazene-level intermediate mechanism predicts that the $K_m(N_2)$ for HD formation should be the same as the $K_m(N_2)$ for NH_3 formation. However, apparent $K_m(N_2)$ values for HD formation calculated from previously published data (Jackson et al., 1968; Bulen, 1976; Newton et al., 1977) or by using our total HD data (Figure 2) are much lower than the apparent $K_m(N_2)$ for ammonia formation (Hwang et al., 1973; Hadfield & Bulen, 1969). Because, under 50% D_2, N_2-independent HD formation accounts for 6% of the electrons that would normally go to H_2 evolution, the two types of HD formation can be quantitated for our N_2-dependence experiment. Figure 5 is a plot of the rates of formation of HD_{total}, N_2-independent HD (calculated as 12% of the H_2 evolved), and N_2-dependent HD (HD_{total} – N_2-independent HD) as a function of p_{N_2}. A double-reciprocal plot of the calculated N_2-dependent HD data (not shown) is linear with an apparent $K_m(N_2)$ for HD formation of 0.12 ± 0.01 atm, which agrees well with previously published $K_m(N_2)$ for NH_3 formation for *Av* nitrogenase (Hwang et al., 1973; Hadfield & Bulen, 1969) and adds further support for the occurrence of HD formation along the normal N_2 reduction pathway.

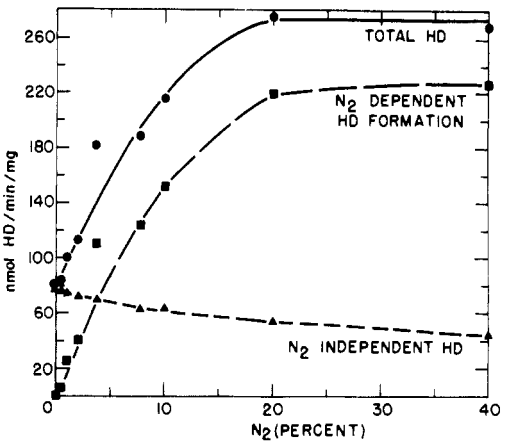
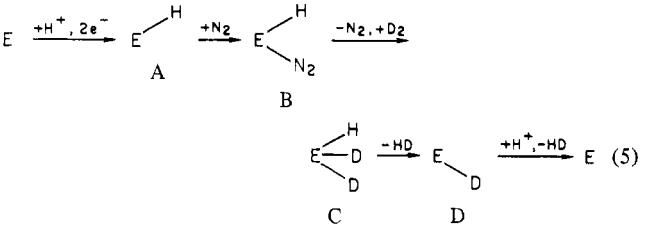


FIGURE 5: Plots of the rate of total HD formation (●), N_2-independent HD formation (▲), and N_2-dependent HD formation (■) as a function of N_2 pressure. N_2-independent HD was calculated from data shown in Figure 2 as ($0.12H_2$), and N_2-dependent HD was calculated as (HD_{total} – N_2-independent HD).

A possible alternative explanation of the HD formation reaction based on hydride–deuteride interchange on a metal center of the enzyme is shown in eq 5. Such a reaction



requires two electrons, two protons, one nitrogen molecule, and one deuterium molecule, in keeping with the observed stoichiometry. This scheme could also explain the ATP-dependent H_2 evolution reaction by protonation of species A as well as H_2 inhibition of N_2 reduction by competition between H_2 and N_2 for species A to form species analogous to B or C (cf. eq 2–3). Its weakness lies in the *requirement* for N_2 to allow D_2 (or H_2) to form the trihydride species C. There is no obvious reason why D_2 (or H_2) should not bind directly to species A without the intermediate species B. But, if binding of N_2 effected some change in the enzyme which then allowed D_2 (or H_2) to displace the N_2 and bind, then the closely related competitive inhibitor and nitrogenase substrate, nitrous oxide (N_2O), might be expected to be active in HD formation. It is not (Hoch et al., 1960; Newton et al., 1977). Further, the deuteride on species D cannot be susceptible to exchange with solvent protons, nor can it be transferred to the bound N_2 molecule during reduction since either of these processes would

allow labeled hydrogen to appear in the solvent in disagreement with the results of our tritium experiment. A mechanism similar to the above hydride–deuteride interchange has recently been proposed by Chatt (1980) based on a trihydride species. However, there are, at least, two major inconsistencies between his proposal and our data. First, a decrease in H_2 evolution is implied in his mechanism when D_2 is added to a N_2-fixing system, such that one H_2 molecule *less* should be evident for every two HD molecules formed. Thus, 37% less H_2 should be evolved on the basis of our HD data under a 50% D_2/40% N_2/10% Ar atmosphere when compared to 40% N_2/60% Ar. In fact, only a 3% decrease in H_2 evolution is observed, which is better explained by N_2-independent HD formation, with the major effect being manifested in decreasing NH_3 formation. Second, this mechanism predicts a *maximum* of one HD formed for every N_2 reduced; that is, of all the electrons used for HD formation plus NH_3 production, a maximum of 17% (one in seven) can appear as HD. Our data, under 50% D_2/40% N_2/10% Ar, show that, in fact, 50% of these electrons can be used to form HD. At this time, we prefer the diazene-level intermediate explanation for the N_2-dependent HD formation reaction. However, a mechanism such as that outlined in eq 5 (minus species B) is a viable possibility for the formation of N_2-independent HD. It should be noted, moreover, that E–H could also represent a protonated ligand of a reduced metal site on the enzyme which can, by a coupled proton–electron transfer process, react in a manner stoichiometrically equivalent to a hydride (Stiefel et al., 1977).

The reduction of N_2 to a diazene-level intermediate implies a mechanism of reduction involving two-electron steps. Carrying this argument one step further leads to the expectation of a hydrazine-level species as the next reduction intermediate. Our results extend Bulen's (Bulen, 1976) earlier findings and firmly establish hydrazine reduction to ammonia as a reaction which is catalyzed by nitrogenase. Thus, hydrazine could possibly be the entity sensitive to H_2 inhibition and responsible for HD formation from D_2 rather than the bound diazene-level intermediate discussed above. However, our results show that hydrazine reduction to ammonia is not inhibited by H_2 and does not enhance HD formation under D_2. If the reduction of added hydrazine is comparable to the reduction of a hydrazine-level intermediate in the N_2-to-ammonia pathway, then the H_2 inhibition and HD formation reactions must occur at an earlier intermediate in this pathway, namely, at the diazene level. Recently, Thorneley et al. (1978) have produced independent evidence for a bound dinitrogen–hydride intermediate in the reduction of N_2 to ammonia. It is important to note that the formation of this intermediate appeared to be inhibited by H_2. The relationship between this intermediate and the ones we have postulated here is not yet established.

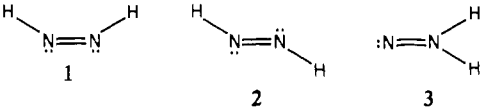
Thermodynamic estimates have been used (Shilov, 1974; Leigh, 1977) to argue against the diazene level as an intermediate in the N_2-to-ammonia pathway. On the basis of the $E^{\circ\prime}$ values for appropriate half-reactions (Table III), the overall nitrogenase reaction of NH_4^+ formation is thermodynamically reasonable ($E^{\circ\prime}$ = −280 mV) and readily effected with $S_2O_4^{2-}$ ($E^{\circ\prime}$ = −386 mV) in vitro or by the putative physiological reductant, flavodoxin ($E^{\circ\prime}$ = −495 mV) without ATP hydrolysis. However, the attainment of the levels of N_2H_2 and N_2H_4 is not thermodynamically favored. This potential thermodynamic limitation may be overcome by (1) the mandatory H_2 evolution reaction, (2) the mandatory ATP hydrolysis, and (3) bound, rather than free, intermediates. The concomitant formation of H_2 and ammonia does not significantly change the thermodynamics for the overall process but could lower $E^{\circ\prime}$ for N_2H_2 production significantly (Table III). Likewise, hydrolysis of MgATP produces −35 kcal/mol which, if tightly coupled to a two-electron transfer, is equivalent to $E^{\circ\prime}$ = −750 mV (Stiefel, 1977) and could alone (or with H_2 evolution) provide sufficient free energy to form the diazene intermediate. Further, if the binding of N_2H_2 (e.g., to a metal center, perhaps enhanced by hydrogen bonding) is stronger than that of N_2, then the $E^{\circ\prime}$ for E–N_2H_2 formation will be substantially less negative than the value for free N_2H_2. Thus, the seemingly large thermodynamic barrier for N_2H_2 formation, and therefore the arguments for precluding the diazene-level intermediate, can be overcome by some combination of the above features.

Table III: $E^{\circ\prime}$ Values for Relevant Half-Reactions

reaction	$E^{\circ\prime}$ (mV)[a]
$N_2(g) + 6e^- + 8H^+ \rightarrow 2NH_4^+(aq)$	−280
$N_2(g) + 2e^- + 2H^+ \rightarrow N_2H_2(aq)$	[−1470][b]
$N_2(g) + 4e^- + 5H^+ \rightarrow N_2H_5^+$	−795
$N_2(g) + 8e^- + 10H^+ \rightarrow 2NH_4^+(aq) + H_2(g)$	−314
$N_2 + 4e^- + 4H^+ \rightarrow N_2H_2 + H_2$	−735
$2H^+ + 2e^- \rightarrow H_2(g)$	−414
$2H^+ + 2e^- + 2HSO_3^- \rightarrow S_2O_4^{2-} + 2H_2O$	−386[c]

[a] Values were from Lattimer (1952) or calculated from free energy data in *NBS Tech. Note (U.S.)* (1968) *No. 270-3* and corrected to pH 7 with the Nernst equation. [b] There is insufficient information available to arrive to a definitive $E^{\circ\prime}$ for $N_2H_2(aq)$ formation. Reported values for $\Delta H^{\circ\prime}$ for the formation of gaseous N_2H_2 [from $H_2(g)$ and $N_2(g)$] are 36 (Willis et al., 1976) and 51 kcal/mol (Foner & Hudson, 1978). Theoretical studies (Kao & Huang, 1979) favor the former value which we use. With the assumption of a reasonable $T\Delta S^\circ$ value (Stiefel, 1977), the ΔG°_f for N_2H_2 is −51 kcal/mol which corresponds to an $E^{\circ\prime}$ estimate of either −1050 mV in 1 M acid solution or −1470 mV at pH 7. The pK_a of $N_2H_3^+(aq)$ is not known, but, if it is assumed to be >7, protonated N_2H_2 would have a less negative value for $E^{\circ\prime}$. [c] From Mayhew (1978).

The reaction of the proposed N_2H_2 intermediate with H_2 (D_2) is simply the decomposition of N_2H_2 to its elements catalyzed by H_2, i.e., $N_2H_2 + H_2 \rightarrow N_2 + 2H_2$. For free N_2H_2, this reaction is expected to be highly exergonic (see Table III), even for bound diazene. Therefore, production of additional H_2 by a N_2-dependent pathway via a N_2H_2-level intermediate is very reasonable and has the same thermodynamic and stoichiometric parameters as ATP-dependent H_2 evolution. The bound N_2H_2 intermediate could take the form of *cis*-diazene (**1**), *trans*-diazene (**2**), or aminonitrene (**3**). It



1 2 3

apparently remains tightly bound during turnover, most probably to the FeMo cofactor site (Shah & Brill, 1977; Cramer et al., 1978; Rawlings et al., 1978; Burgess et al., 1980b) of the molybdenum–iron protein of nitrogenase. We presently prefer bound *cis*-N_2H_2 as the target of H_2 (D_2) interception on the basis of the formation of *cis*-$C_2H_2D_2$ from C_2H_2 in D_2O. Further, the cis species is compatible with the formation of a cyclic transition state or intermediate in the H_2 inhibition/HD production reaction (Newton et al., 1977; Stiefel et al., 1977).

In conclusion, the data presented herein firmly establish that (1) HD formation is a property of nitrogenase, (2) HD is formed by two separate pathways, one being dependent on N_2 and the other N_2 independent, (3) tritium and, by implication, D_2 exchange with solvent protons is not catalyzed by nit-

rogenase, (4) one electron is consumed for each HD molecule formed, (5) N_2-dependent HD formation and H_2 (D_2) inhibition of N_2 reduction occur by the same mechanism, and (6) hydrazine is a substrate of nitrogenase which is not sensitive to H_2 and does not form HD from D_2. These observations may be interpreted in terms of reaction at the diazene level during N_2 reduction. The dissection of the six-electron N_2-reduction process into two-electron steps appears as a minimum requirement in our interpretation of the current results. Whether the various two-electron, two-proton steps can be further decomposed into single-proton and/or single-electron steps is a question toward which future studies will be directed.

Acknowledgments

We thank Dr. J. L. Corbin for providing creatine phosphate and Dr. G. D. Watt for help with the $S_2O_4^{2-}$ utilization studies.

Supplementary Material Available

Two tables listing data on N_2 and hydrazine concentration dependence (1 page). Ordering information is given on any current masthead page.

References

Antanol, J. (1961) French Patent 1 211 099.
Bergerson, F. J. (1963) *Aust. J. Biol. Sci. 16*, 669–680.
Bulen, W. A. (1976) *Proc. Int. Symp. Nitrogen Fixation, 1st, 1974 1*, 177–186.
Bulen, W. A., & LeComte, J. R. (1966) *Proc. Natl. Acad. Sci. U.S.A. 56*, 979–986.
Bulen, W. A., & LeComte, J. R. (1972) *Methods Enzymol. 24B*, 456–470.
Bulen, W. A., Burns, R. C., & LeComte, J. R. (1965) *Proc. Natl. Acad. Sci. U.S.A. 53*, 532–539.
Burgess, B. K., Wherland, S., Stiefel, E. I., & Newton, W. E. (1980a) in *Molybdenum Chemistry of Biological Significance* (Newton, W. E., & Otsuka, S., Eds.) pp 73–84, Plenum Press, New York.
Burgess, B. K., Stiefel, E. I., & Newton, W. E. (1980b) *J. Biol. Chem. 255*, 353–356.
Burgess, B. K., Jacobs, D. B., & Stiefel, E. I. (1980c) *Biochim. Biophys. Acta 614*, 196–209.
Burris, R. H., Winter, H. C., Munson, T. O., & Garcia-Rivera, J. (1965) in *Non-Heme Iron Proteins, Role in Energy Conservation* (San Pietro, A., Ed.) pp 315–321, Antioch Press, Yellow Springs, OH.
Chatt, J. (1980) in *Nitrogen Fixation* (Stewart, W. D. P., & Gallon, J. R., Eds.) pp 1–17, Academic Press, London.
Cramer, S. P., Gillum, W. O., Hodgson, K. O., Mortenson, L. E., Stiefel, E. I., Chisnell, J. R., Brill, W. J., & Shah, V. K. (1978) *J. Am. Chem. Soc. 100*, 3814–3819.
Evans, H. J., Emerich, D. W., Ruiz-Argüeso, T., Maier, R. J., & Albrecht, S. L. (1980) in *Nitrogen Fixation* (Newton, W. E., & Orme-Johnson, W. H., Eds.) Vol. 2, pp 69–86, University Park Press, Baltimore, MD.
Foner, S. N., & Hudson, R. L. (1978) *J. Chem. Phys. 68*, 3162–3168.
Hadfield, K. L., & Bulen, W. A. (1969) *Biochemistry 8*, 5103–5108.
Hoch, G. E., Schneider, K. C., & Burris, R. H. (1960) *Biochim. Biophys. Acta 37*, 273–279.
Hwang, J. L., Chen, C. H., & Burris, R. H. (1973) *Biochim. Biophys. Acta 292*, 256–270.
Jackson, E. K., Parshall, G. W., & Hardy, R. W. F. (1968) *J. Biol. Chem. 243*, 4952–4958.
Kao, J., & Huang, T.-N. (1979) *J. Am. Chem. Soc. 101*, 5546–5557.
Kelly, M. (1968) *Biochem. J. 109*, 322–324.
Lattimer, W. (1952) *Oxidation Potentials*, 2nd ed., Prentice Hall, Englewood Cliffs, NJ.
Leigh, G. J. (1977) *Recent Dev. Nitrogen Fixation*, [*Proc. Int. Symp.*], *2nd, 1976*, 1–24.
Mayhew, S. G. (1978) *Eur. J. Biochem. 85*, 535–547.
NBS Tech. Note (*U.S.*) (1968) *No. 270-3*.
Newton, W. E., Corbin, J. L., & McDonald, J. W. (1976) *Proc. Int. Symp. Nitrogen Fixation, 1st, 1974 1*, 53–74.
Newton W. E., Bulen, W. A., Hadfield, K. L., Stiefel, E. I., & Watt, G. D. (1977) *Recent Dev. Nitrogen Fixation*, [*Proc. Int. Symp.*], *2nd, 1976*, 119–130.
Rawlings, J., Shah, V. K., Chisnell, J. R., Brill, W. J., Zimmerman, R., Münck, E., & Orme-Johnson, W. H. (1978) *J. Biol. Chem. 253*, 1001–1004.
Rivera-Ortiz, J. M., & Burris, R. H. (1975) *J. Bacteriol. 123*, 537–545.
Shah, V. K., & Brill, W. J. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 3249–3253.
Shilov, A. E. (1974) *Russ. Chem. Rev.* (*Engl. Transl.*) *43*, 378–398.
Stiefel, E. I. (1977) *Recent Dev. Nitrogen Fixation*, [*Proc. Int. Symp.*], *2nd, 1976*, 69–108.
Stiefel, E. I., Newton, W. E., Watt, G. D., Hadfield, K. L., & Bulen, W. A. (1977) *Adv. Chem. Ser. No. 162*, 353–388.
Stiefel, E. I., Burgess, B. K., Wherland, S., Newton, W. E., Corbin, J. L., & Watt, G. D. (1980) in *Nitrogen Fixation* (Newton, W. E., & Orme-Johnson, W. H., Eds.) Vol. 1, pp 211–222, University Park Press, Baltimore, MD.
Strandberg, G. W., & Wilson, P. W. (1967) *Proc. Natl. Acad. Sci. U.S.A. 58*, 1404–1409.
Thorneley, R. N. F., Eady, R. R., & Lowe, D. J. (1978) *Nature* (*London*) *272*, 557–558.
Turner, G. L., & Bergerson, F. J. (1969) *Biochem. J. 115*, 529–535.
Watt, G. D., & Burns, A. (1977) *Biochemistry 16*, 265–270.
Wherland, S., Burgess, B. K., Stiefel, E. I., & Newton, W. E. (1981) *Biochemistry* (preceding paper in this issue).
Willis, C., Lossing, F. P., & Back, R. A. (1976) *Can. J. Chem. 54*, 1–3.
Wilson, P. W., & Umbreit, W. W. (1937) *Arch. Mikrobiol. 8*, 440–457.