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The Chemical Evolution of a Nitrogenase Model. VII. The Reduction of Nitrogen

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Abstract: Nitrogenase model systems composed of molybdate and thiol ligands such as L(+)-cysteine reduce molecular nitrogen to ammonia slowly in the presence of $NaBH_4$ as the reducing agent. In the presence of substrate amounts of ATP, the reduction of nitrogen is significantly stimulated but leads to the accumulation of diimide in the reaction solutions. The diimide decomposes or disproportionates to nitrogen, hydrogen, and hydrazine and is not reduced as such. The ammonia produced in all reactions arises primarily from the reduction of hydrazine. These observations were confirmed by independent experiments with diimide generated by the decomposition of azodicarboxylate and with hydrazine. Diimide is not a substrate for molybdothiol catalyst systems, while hydrazine is catalytically reduced to ammonia. Molybdothiol catalysts promote D_2–H^+ exchange most efficiently in the presence of nitrogen as the substrate; they thus parallel nitrogenase also in this respect. The D_2–H^+ exchange reaction is presumably linked to the formation and decomposition of diimide in both the nitrogenase enzyme and its models. The current molybdothiol model systems were furthermore expanded to include ferredoxin-type complexes as electron-transfer catalysts. This provided new catalytically active systems containing molybdenum, iron, labile sulfide, and RSH components in proportions similar to that observed in native nitrogenase. The new model systems catalyze the reduction, of, *e.g.*, acetylene efficiently even with $S_2O_4^{2-}$ as the reducing agent and thus duplicate nitrogenase in yet another important respect.

In previous papers[1-7] we reported on the reduction of known alternate substrates of nitrogenase (N_2-ase) with nonenzymatic model systems composed of molybdate, a thiol ligand such as 1-thioglycerol, or, in most cases, L(+)-cysteine, using $NaBH_4$ or $Na_2S_2O_4$ as reducing agents. The behavior of these homogeneous catalyst systems resembles that of N_2-ase. In particular, the reactions with reducible substrates are significantly stimulated by ATP. Their ability to reduce molecular nitrogen was also demonstrated, initially at elevated pressures using $^{28}N_2$[1,4] and later at 1 atm with $^{30}N_2$ as the substrate.[6,8] In the present paper we describe further results of the reactions with nitrogen, as well as an extension of the model systems to include ferredoxin model compounds as electron-transfer catalysts. We will also report the results of D_2–H^+ exchange experiments, as well as the catalysis of hydrazine reduction by our systems.

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(2) G. N. Schrauzer, *Advan. Chem. Ser.*, **No. 100**, 1 (1971).
(3) G. N. Schrauzer and P. A. Doemeny, *J. Amer. Chem. Soc.*, **93**, 1608 (1971).
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(6) G. N. Schrauzer, P. A. Doemeny, R. H. Frazier, and G. W. Kiefer, *J. Amer. Chem. Soc.*, **94**, 7378 (1972).
(7) G. N. Schrauzer, G. W. Kiefer, P. A. Doemeny, and H. Kisch, *J. Amer. Chem. Soc.*, **95**, 5582 (1973).
(8) R. E. E. Hill and R. L. Richards, *Nature (London)*, **233**, 114 (1971).

Results

Model Systems, Substrates, and Assays Employed. The first experiments with nitrogen as the substrate were performed with catalysts composed of molybdate and 1-thioglycerol.[1,4] The latter was employed primarily to avoid any contamination of the system by introducing ammonia from the ligands. This occurs, for example, when 2-aminoethanethiol is used[6] and has led to an overestimation[8] of the yield of ammonia produced in reactions with molecular nitrogen and complexes of molybdenum containing this ligand. In most of the work to be reported in the present paper we have used our standard nitrogenase model system, which consists of the binuclear molybdenum(V) complex of composition $Na_4Mo_2O_4(Cys)_2$ in aqueous solution. This compound, hereinafter designated complex I,

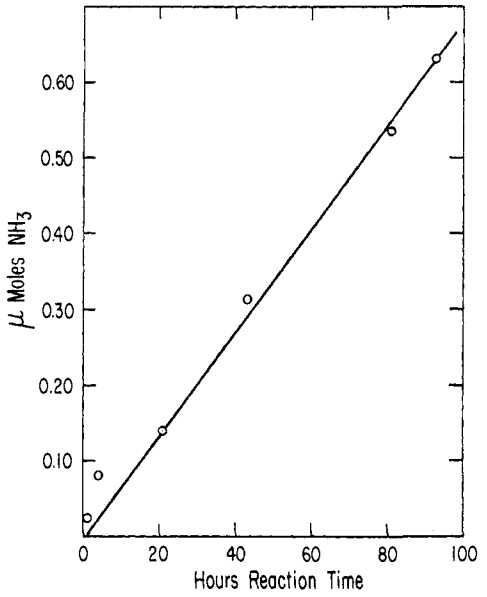


Figure 1. Formation of $^{15}NH_3$ from $^{30}N_2$ at 1 atm of partial pressure in solutions containing complex I and $NaBH_4$. Reaction vials of 30-ml capacity contained 0.024 mmol of complex I, $[NaBH_4]_{init}$ = 0.16 *M* in pH 9.6 borate buffer (0.2 *M*). The total reaction volume is 4 ml.

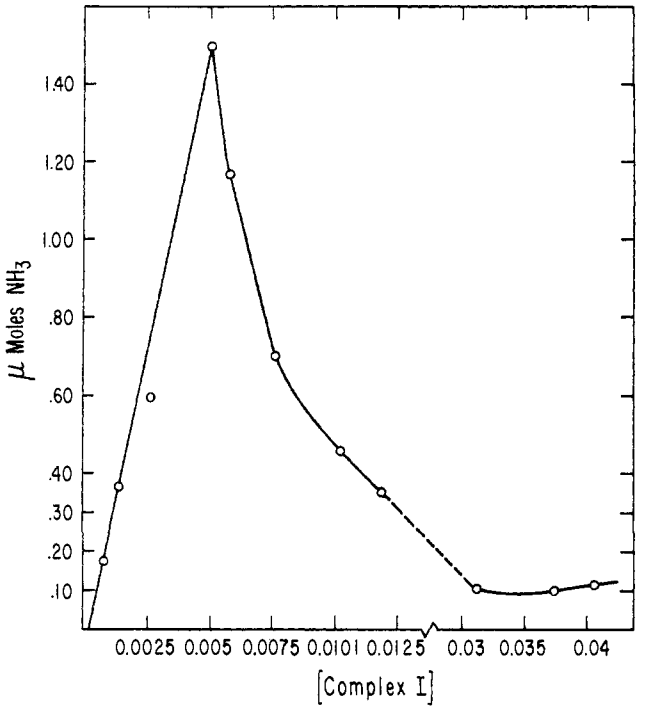


Figure 2. Dependence of the yield of $^{15}NH_3$ after 92 hr of reaction at 27° on the concentration of complex I. The total reaction volume is 3.5 ml; $[NaBH_4]_{init}$ = 0.16 *M*, in 0.2 *M* pH 9.6 borate buffer. For results under slightly different reaction conditions also see Table I.

undergoes further reaction under reducing conditions, producing monomeric Mo–Cys species which are the actual catalysts. Cysteine is relatively stable in our system at room temperature and releases little if any ammonia unless the reaction solutions are heated. However, to avoid possible interference with intrinsic ammonia, all experiments were performed with $^{30}N_2$ as the substrate. The reaction solutions were either analyzed as such or distilled prior to the assay for $^{15}NH_3$ in all cases by using the standard hypobromite oxidation technique.[9] The amount of unlabeled ammonia introduced with the reagents was estimated to *ca.* 1 μmol. Ammonia formed from $^{30}N_2$ would accordingly give rise to a mixture of $^{30}N_2$ and $^{29}N_2$; the ratio between the two isotopes of nitrogen would vary as a function of the concentration of background ammonia present in the system. It was for this reason preferable to add a small amount (10–50 μmol) of unlabeled ammonium chloride to the reaction solutions, to assure that all of the $^{15}NH_3$ produced would be converted to $^{29}N_2$ on hypobromite oxidation. This in turn permitted a distinction of ammonia from any precursors of ammonia in which the original N–N bond of the substrate was still present, *i.e.*, from diimide and hydrazine; if these are formed from $^{30}N_2$, hypobromite oxidation will convert them back into $^{30}N_2$. The yields were determined by mass spectrographic analysis. All results quoted are corrected for natural abundance of ^{15}N by subtracting the calculated amount of $^{29}N_2$ from the observed $^{29}N_2$ yields. The reaction solutions were always thoroughly degassed prior to hypobromite oxidation, and the background levels of $^{29}N_2$ and $^{30}N_2$ were monitored in each experimental series. The yields of product were obtained from calibration runs in which a known amount of $^{15}NH_4Cl$ was added to reaction solutions, followed by hypobromite oxidation. Ammonia and hydrazine were in some cases also determined by spectrophotometric assay procedures described in the Experimental Section.

(9) R. F. Glascock in "Isotopic Gas Analysis," Academic Press, New York, N. Y., 1954, p 195.

Nitrogen Fixation in the Absence of ATP. Molecular nitrogen at 1 atm of partial pressure reacts with complex I–$NaBH_4$ to yield ammonia at an exceedingly slow rate. After 100 hr of reaction at ambient temperature, 0.65 μmol of NH_3 is produced from 0.024 mmol of complex I (Figure 1), corresponding to the approximate turnover of 0.1 mmol of N_2 per hour per mole of molybdenum. The yield of ammonia increases approximately linearly during the first 100 hr of reaction, but there appears to be a deviation from linearity at the time points after 1 and 3 hr of reaction. The further investigation of this phenomenon, which is even more pronounced in the reactions in the presence of ATP, led to the detection of a labile precursor of ammonia (see below). The yield of ammonia depends on the concentration of complex I in a manner similar to that observed in the experiments with other substrates[5,6] and is shown in Figure 2. The observed dependence of the yield on [complex I] reflects primarily the concentration dependence of equilibria between monomeric and dimeric forms of the Mo–Cys complexes and suggests that the catalytically active species are mononuclear, as in the reduction of the other substrates. At best insubstantial increases in the yield of ammonia were observed in the presence of cocatalytic amounts of iron (added in the form of $FeSO_4 \cdot 7H_2O$), at least as long as complex I is used as the catalyst. In reaction systems containing MoO_4^{2-} and Cys in the molar ratio of 1:1, iron seems to have a more significant positive effect, but the overall catalytic efficiency is lower than that of systems containing complex I. Numerical data for nitrogen reduction experiments under various conditions in the absence of ATP are summarized in Table I.

Table I. Nitrogen Fixation with Complex I–$NaBH_4$ in the Absence of ATP[a]

No.	[Complex I], mol/l.	Time, hr	NH_3, μmol
1	0.006	0	0
2	0.006	1	0.024
3	0.006	3	0.082
4	0.006	20	0.14
5	0.006	43	0.33
6	0.006	72	0.48
7	0.006	92	0.63
8	0.006	116	0.98
9	0.00075	72	0.06
10	0.0015	72	0.08
11	0.003	72	0.12
12	0.02	95	0.72
13	0.02	252	1.35
14	0.06	122	0.33
15	0.24	116	0.99
16	0.006 + Fe^{2+}	0	0
17	0.006 + Fe^{2+}	3	0.05
18	0.006 + Fe^{2+}	12	0.18
19	0.006 + Fe^{2+}	20	0.22
20	0.006 + Fe^{2+}	43	0.28

[a] Substrate: 99+% $^{30}N_2$; reaction temperature, 27°; total reaction volume, 4.0 ml; initial [$NaBH_4$] = 0.15 *M*; in pH 9.6 borate buffer (0.2 *M*).

Effect of ATP. The addition of ATP causes a significant stimulation of the activity of complex I and related catalysts in the reduction of all alternate substrates of N_2-ase. In the case of nitrogen as the substrate it appeared to have an adverse effect, however, suggesting an anomalous behavior of nitrogen as compared to the other substrates. Thus, only very small amounts of ^{15}N were present after 40–100 hr of reaction. The effect of ATP was initially attributed to its accelerating effect on borohydride decomposition, leading to a more rapid loss of hydridic activity in the systems. The addition of ATP to mixtures of complex I with $NaBH_4$ is accompanied by a burst of hydrogen evolution, and virtually all of the BH_4^- is decomposed within 40 min. In the absence of ATP the pH 9.6 buffered solutions of complex I and $NaBH_4$ retain hydridic activity for at least 48 hr. This observation led us to investigate the time dependence of nitrogen fixation in greater detail.

Identification of Diimide. The reaction of $^{30}N_2$ with complex I, $NaBH_4$ in the presence of, *e.g.*, 0.60 mmol of ATP, causes the initial rapid formation of an unstable precursor of ammonia, which accumulates during the first 40 min of reaction and subsequently gradually disappears. This follows from the data represented in Figure 3, which indicates the yields of $^{30}N_2$ and $^{29}N_2$ in the reaction solutions before and after distillation, after hypobromite oxidation. It may be seen that $^{30}N_2$ is formed in much higher yields than $^{29}N_2$. Hence, the main product of the reduction of nitrogen under these conditions was not ammonia, since this should have given rise to the nearly exclusive formation of $^{29}N_2$ (the reaction solutions contained added $^{14}NH_4^+$ to trap all $^{15}NH_4^+$ in the form of $^{29}N_2$ on hypobromite oxidation). The precursor of ammonia was identified as diimide, N_2H_2, on the basis of the following points of evidence.

(a) Using 1:1 mixtures of $^{28}N_2$ and $^{30}N_2$ as the substrate causes a lowering of the yield of $^{30}N_2$ released on hypobromite oxidation to one-half of the amount formed when the substrate was pure $^{30}N_2$. The yield of $^{29}N_2$ also decreases. This demonstrates that the precursor contains the original N–N bond of the substrate (see Table II).

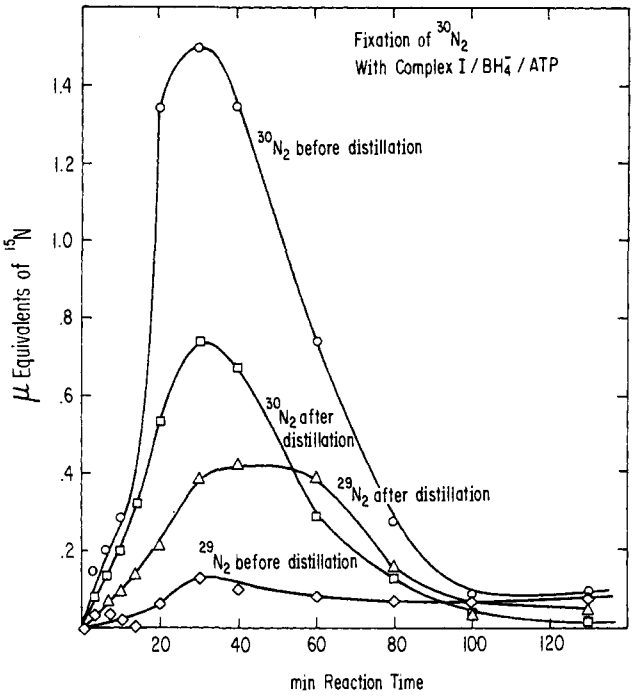


Figure 3. Variations of the yields of $^{30}N_2$ and $^{29}N_2$ on hypobromite oxidation of reaction solutions at various time points. Reaction solutions contained 0.024 mmol of complex I, 0.05 mmol of $^{14}NH_4Cl$ (internal marker for trapping $^{15}NH_3$ as $^{29}N_2$ after hypobromite oxidation), $[ATP]_{init}$ = 0.15 *M*; $[NaBH_4]_{init}$ = 0.16 *M*; total solution volume = 4.0 ml; reaction temperature = 27°.

Table II. Yields of $^{30}N_2$ of $^{29}N_2$ (μAtom Equivalents) with 99+% $^{30}N_2$ (A) and a 1:1 Mixture with $^{28}N_2$ (B), after Hypobromite Oxidation of the Reaction Solutions[a]

Reaction time, min	A		B	
	$^{30}N_2$	$^{29}N_2$	$^{30}N_2$	$^{29}N_2$
20	1.33	0.04	0.59	0.02
30	1.48	0.06	0.70	0
40	1.32	0.08	0.60	0.05
60	0.72	0.07	0.38	0.04

[a] Experimental conditions as given in Figure 3.

(b) Distillation of previously acidified alkaline reaction solutions containing ^{15}N-labeled precursor lowers the yield of $^{30}N_2$ after hypobromite oxidation to a varying extent, in most cases to one-half of the original amount (Table III). This is consistent with the disproportionation of diimide to nitrogen and hydrazine.

$$2N_2H_2 \longrightarrow N_2H_4 + N_2 \quad (1)$$

Using the colorimetric assay, hydrazine was detected in the distillates.

(c) The addition of an excess of olefinic substrates such as sodium maleate or *cis*-4,5-cyclohexenedicarboxylate to reaction solutions containing the diimide induces the oxidation of diimide to nitrogen (Table IV). The addition of sodium succinate or of 1,2-cyclohexanedicarboxylate has no effect. Diimide is known[10] to react with olefinic substrates according to eq 2.

(10) (a) S. Hünig, H. R. Müller, and W. Thier, *Angew. Chem.*, 77, 368 (1965); *Angew. Chem., Int. Ed. Engl.*, **4**, 271 (1965); (b) C. E. Miller, *J. Chem. Educ.*, **42**, 254 (1965); (c) N. Wiberg, H. Bachhuber, and G. Fischer, *Angew. Chem.*, **84**, 889 (1972); *Angew. Chem., Int. Ed. Engl.*, **11**, 829 (1972).

Table III. Yields of $^{30}N_2$ and of $^{29}N_2$ (μAtom Equivalents) before and after the Distillation of Previously Acidified Reaction Solutions[a]

Reaction time, min	Before distillation $^{30}N_2$	Before distillation $^{29}N_2$	After distillation $^{30}N_2$	After distillation $^{29}N_2$	% recovery of ^{15}N on distillation
0	0	0	0	0	
3	0.14	0.03	0.08	0.03	59.4
6	0.20	0.03	0.13	0.06	72.7
10	0.28	0.02	0.20	0.10	86.2
14	0.71	0	0.35	0.13	59.2
20	1.33	0.05	0.55	0.21	48.5
30	1.48	0.10	0.77	0.38	62.7
40	1.32	0.08	0.67	0.41	64.7
60	0.72	0.075	0.28	0.41	63.1
80	0.27	0.07	0.13	0.17	70.9
100	0.10	0.07	0.06	0.08	71.4
130	0.11	0.07	0.03	0.09	53.3

[a] Experimental conditions as given in Figure 3.

Table IV. Effect of Diimide Trapping Agents on the Yields of $^{30}N_2$ and $^{29}N_2$ Released on Hypobromite Oxidation[a]

No.	Additive and conditions[b]	$^{30}N_2$	$^{29}N_2$, μequiv
1	None, after 40 min of reaction	1.48	0.07
2	Maleate	0.025	0.01
3	Maleate added after 40 min of reaction	0	0.07
4	CHD^{2-} [c]	0.215	0.019
5	CHD^{2-}, added after 40 min of reaction	0.143	0.04
6	Succinate	0.85	0.05
7	Succinate, added after 60 min of reaction	0.70	0.01
8	None, after 60 min of reaction	0.72	0.005

[a] Reaction conditions as in Figure 3, except for added excess of the trapping agents. [b] All additives were the sodium salts at concentrations of 0.2 *M*. [c] CHD^{2-} is *cis*-4,5-cyclohexenedicarboxylate.

$$N_2H_2 + RCH{=}CHR \longrightarrow \text{(diimide)} \longrightarrow N_2 + C_2H_4R_2 \quad (2)$$

Factors Influencing the Yield of Diimide. The known instability and high reactivity of diimide causes the yields of diimide in our system to be strongly dependent on details of the reaction conditions. We usually stopped our reactions by adding concentrated HCl at room temperature. However, when the reaction solutions were cooled prior to acidification, a higher yield of diimide was observed. The yields of diimide were unchanged if the reactions were conducted in 0.2 *M* pH 9.6 EDTA buffer, instead of borate. However, significantly higher yields were observed in 0.2 *M* pH 9.6 and 7.0 phosphate buffer. The addition of excess $NaBH_4$ and of cocatalytic amounts of Cu^{2+} or Fe^{2+} salts to the reaction solutions containing preformed diimide causes a nearly quantitative decomposition (Table V). This demonstrates the sensitivity of diimide in the presence of oxidizing metals or catalysts. Neither copper nor iron catalyzes the reduction of diimide to ammonia in alkaline or acidic solutions. Finally, the yields of diimide and ammonia are higher in D_2O as the reaction medium (Table V).

K_m Value and Turnover Number for N_2 Reduction to Diimide. The K_m value for N_2 with respect to reduction to N_2H_2 with the standard N_2-ase model system was determined from rate measurements at $^{30}N_2$ pressures between 0 and 1 atm. From five available experimental points the K_m value was calculated to 0.5 ± 0.2 atm at 27°. From the initial rates of diimide formation the turnover number for N_2 reduction was estimated to be in the order of 3 (millimoles of N_2 reduced per mole of molybdenum per min) in pH 9.6 borate buffer and 5 in pH 9.6 phosphate buffer (uncorrected for diimide decomposition).

Table V. Factors Influencing the Yield of Diimide and Its Reduction to Ammonia

No.	Conditions[a]	μEquiv N_2H_2	μEquiv NH_3	% N_2H_2 reduced to NH_3
1	pH 9.6 borate buffer, 27°, during acidification	0.72	0.0015	0.2
2	pH 9.6 borate buffer, 8°, during acidification	0.91	0.0007	0.08
3	pH 9.6 phosphate buffer	1.80	0.025	1.5
4	pH 7.0 phosphate buffer	2.30	0.035	1.5
5	pH 4.0 phosphate buffer	1.24	0.020	1.6
6	pH 9.6 borate buffer, plus added Cu^{2+} and $NaBH_4$, acidified	0.04	0.007	0.1
7	pH 9.6 borate buffer, plus added Fe^{2+} and $NaBH_4$	0	0.003	0.5
8	pH 9.6 borate buffer in D_2O, 27°, during acidification	0.95	0.05	5

[a] The reaction solutions contained complex I, ATP, and $NaBH_4$ in initial amounts as given in the legend of Figure 3; all solutions were stopped after 60 min of reaction.

Effect of Other Nucleoside Phosphates. Since the model system is nonselective with respect to ATP and is also stimulated by GTP, ADP, and AMP, these agents were also tested for their effect on diimide formation. The results summarized in Table VI indicate a similar dependence of the yield of diimide as a function of the nucleoside phosphate as compared to the yields of product(s) in the reduction of other substrates. This demonstrates that the nucleoside phosphates stimulate the efficiency of the catalyst independent of the nature of the substrate, in agreement with previous conclusions.[7] In the case of nitrogen, additional complications arise due to the sensitivity of diimide and the ability of certain agents (*e.g.*, phosphates) to retard its decomposition.

Table VI. Relative Yields of Diimide as a Function of Added Nucleoside Phosphates and Related Compounds

	Relative yield of N_2H_2 after 5 min[a]
ATP	100
ADP	87.0
AMP	57.5
Adenosine	0
Adenine	0
GTP	100

[a] In pH 9.6, 0.2 *M* phosphate buffer; reaction conditions as given in Figure 3. The initial concentration of all nucleoside phosphates was 0.15 *M*.

Effect of Ligands on the Diimide Production. A 4:1 molybdenum complex of reduced glutathione, prepared by the method of Werner, Russell, and Evans,[11] catalyzes the reduction of nitrogen to ammonia slowly in the

(11) D. Werner, S. A. Russell, and H. J. Evans, *Proc. Nat. Acad. Sci. U. S.*, **70**, 339 (1973).

absence of ATP, at rates approximately equal to those of complex I based on the amount of molybdenum present. The glutathione complex also catalyzes the reduction of nitrogen to diimide in the presence of substrate amounts of ATP. (Previous claims[11] that this complex does not react with nitrogen must be revised and were presumably due to the insensitivity of the ammonia assay employed.) Other thiol ligands may be used to replace Cys in molybdate-promoted catalytic reactions, but this does not lead to substantial improvements of efficiency. In the MoO_4^{2-}–Cys reactions with molecular nitrogen, cocatalytic amounts of iron as well as substrate amounts of ATP appear to have weak stimulatory effects, as follows from the data compiled in Table VII. Systems composed of bovine serum albumin and MoO_4^{2-} are marginally active.

Table VII. Nitrogen Fixation with Molybdenum Complexes of Various Sulfur Ligands (Substrate, 99+% $^{30}N_2$ at 1 atm of Partial Pressure)[a]

No.	Catalyst[b]	Reaction time	Product μequiv N_2H_2	Product μequiv NH_3
1	Complex I (0.024 mmol)	100 hr	*d*	0.91
2	Mo–GSH (0.012 mmol)	100 hr	*d*	0.72
3	Complex I (0.024 mmol) + ATP	40 min	1.52	0.15
4	Mo–GSH (0.012 mmol) + ATP	40 min	0.77	0.05
5	Complex I (0.024 mmol) + ATP	120 min	0.112	0.005
6	Mo–GSH (0.012 mmol) + ATP	120 min	0.045	0.03
7	MoO_4^{2-}–Cys (1:1) (0.020 mmol)	42 hr	*d*	0.76
8	MoO_4–AET (1:1) (0.020 mmol)	42 hr	*d*	0.66
9	MoO_4^{2-} (0.020 mmol) + 40 mg BSA	42 hr	*d*	0.14
10	MoO_4^{2-}–Cys (2:1)[c]	42 hr	*d*	0.20
11	MoO_4^{2-}–AET (2:1) + Fe^{2+} [c]	42 hr	*d*	0.25
12	MoO_4^{2-}–Cys (2:1) + ATP + Fe^{2+} [c]	42 hr	*d*	1.05
13	MoO_4^{2-}–AET (2:1) + ATP + Fe^{2+} [c]	42 hr	*d*	0.97

[a] Abbreviations: GSH = reduced glutathione; AET = 2-aminoethanethiol; BSA = bovine serum albumin. [b] Total reaction volume 3.5 ml in experiments no. 1–8, 1.8 ml in experiments no. 9–13, in pH 9.6 borate buffer (0.2 *M*). Initial amount of $NaBH_4$, 0.61 mmol. [c] Conditions similar to those reported in ref 8 but in 0.2 *M* pH 9.6 borate buffer. [d] Diimide is usually absent in solutions after long reaction times.

Inhibitors of Diimide Formation. The formation of diimide from N_2 is weakly inhibited by O_2, CN^-, or C_2H_2. Carbon monoxide actually increases the yield, suggesting that it retards the decomposition of diimide under the reaction conditions. Cyanide has a similar effect. Thus, if cyanide ion is added to reaction solutions after 60 min, almost all of the ^{15}N originally present is recovered on hypobromite oxidation. Carbon monoxide retards the decomposition of diimide to a lesser extent and EDTA not at all (Table VIII). Hydrogen does not seem to inhibit diimide formation since it is present in all experiments with $NaBH_4$ as the reducing agent, often at pressures exceeding 5 atm.

Reduction of Diimide to Ammonia. The accumulation and subsequent disappearance of diimide in the reaction solutions of our experiments with A TPindicates that its reduction to hydrazine and ammonia is inefficient, competing unfavorably with its decomposition. Diimide is known to be short lived in aqueous solution, decomposing primarily into nitrogen and hydrogen or disproportionating into nitrogen and hydrazine.[10] In our reaction solutions the concentration of diimide is 10^{-3}–10^{-4} *M*; accordingly, most of the diimide is expected to decompose rather than to disproportionate, since the decomposition into nitrogen and hydrogen is a monomolecular reaction, whereas the disproportionation is bimolecular. The results in Table III indicate that distillation of the reaction solutions containing diimide leads to a recovery of 53–86% of the ^{15}N present. If all of the ^{15}N would have been in the form of diimide, the recovery could have been only 50% or less. On the other hand, some diimide disproportionation occurs undoubtedly even during the reaction and the subsequent experimental operations and it may be expected that our reaction solutions always contain a mixture of diimide and hydrazine. The addition of fresh $NaBH_4$ + complex I reduces only the hydrazine originally present and whatever hydrazine is formed during the reduction by the disproportionation of diimide but not diimide itself. The yields of ammonia vary between 30 and 60% of the amount of diimide originally present and were the highest if the reaction solutions were first acidified, allowed to stand for 60 min, and subsequently reduced by $NaBH_4$–complex I. The observed conversions of diimide to ammonia varied between 10 and 70% of the original amount of ^{15}N present, indicating that only the hydrazine, but not the diimide, was being reduced. This result also suggested that diimide itself is apparently not a substrate, while hydrazine is catalytically reduced. These observations were in turn confirmed by independent experiments using chemically generated diimide as well as hydrazine as the reactants in our systems under various conditions (see below).

Table VIII. Effects of Actual or Potential Inhibitors on the Formation of Diimide from $^{30}N_2$[a]

No.	Inhibitor and reaction conditions	N_2H_2, μequiv
1	None, pH 9.6 borate buffer, after 60 min	0.72
2	None, pH 9.6 EDTA buffer, after 60 min	0.73
3	CO–$^{30}N_2$ (1:1), pH 9.6 borate[b]	2.01
4	C_2H_2–$^{30}N_2$ (1:1), 9.6 borate[b]	0.64
5	CN^- [b]	0.62
6	O_2–$^{30}N_2$ (1:1), pH 9.6 borate[b]	0.58
7	None, after 100 min	0.05
8	EDTA buffer, after 100 min[c]	0.05
9	CN^-, after 100 min[c]	0.75
10	CO, after 100 min[c]	0.25

[a] Concentration of reactants as given in the legend of Figure 3. [b] Inhibitor present at $t = 0$. Solutions assayed after 60 min of reaction. [c] Inhibitor added after 60 min of reaction.

Ferredoxin Model Compounds as Electron-Transfer Catalysts. We have originally approximated the electron-transfer system of N_2-ase simply by adding cocatalytic amounts of Fe^{2+} salt to the molybdothiol catalysts. The added iron proved to be effective only as long as $NaBH_4$ was used as the reductant but not with $Na_2S_2O_4$. Since the latter is normally employed as the reducing agent in *in vitro* experiments with N_2-ase, it appeared desirable to find conditions for its utilization in the molybdothiol model systems. The recently described ferredoxin model compounds[12,13] were investigated first as potential electron-transfer catalysts

(12) B. A. Averill, T. Herskovitz, R. H. Holm, and J. A. Ibers, *J. Amer. Chem. Soc.*, **95**, 3523 (1973).

(13) T. Herskovitz, B. A. Averill, R. H. Holm, J. A. Ibers, W. D. Phillips, and J. F. Weiher, *Proc. Nat. Acad. Sci. U. S.*, **69**, 2437 (1972).

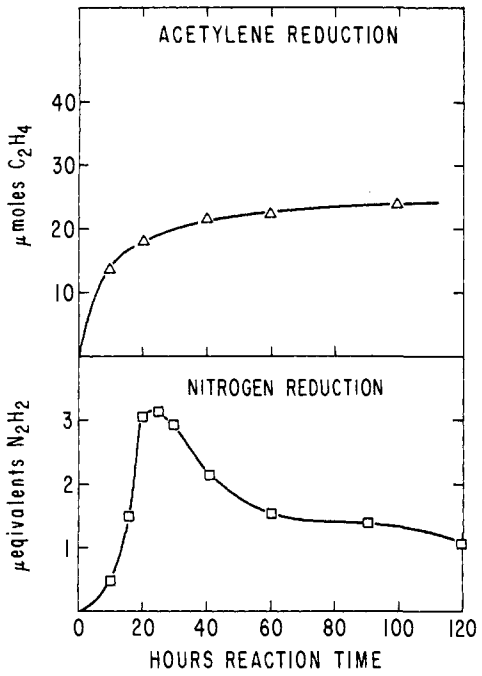


Figure 4. Reduction of acetylene and nitrogen by complex I in the presence of $Fe_4S_4(S\text{-}n\text{-}C_3H_7)_4^{2-}$, with $Na_2S_2O_4$ as the reducing agent. Reaction vials of 30-ml capacity contained 0.024 mmol of complex I; 0.048 mmol of the iron mercaptosulfide tetramer; and $[S_2O_4^{2-}]_{init}$ = 0.125 *M* in 0.1 *M* pH 9.6 borate buffer in 1:1 water–methanol; reaction temperature = 25°; total reaction volume = 4 ml. Both $^{30}N_2$ and C_2H_2 at 1 atm of partial pressure.

and were found to be excellently suited for our purposes. A variety of complex salts of composition $[N(n\text{-}C_4H_9)_4^+]$-$[Fe_4S_4(SR)_4]^{2-}$ (R = alkyl or substituted alkyl) were prepared and tested for catalytic activity in the reduction of acetylene. All were found to be inactive, unless a trace of complex I was added. The ability of the ferredoxin model compounds to transfer electrons to the molybdenum catalysts enables us to work with model systems containing molybdenum, iron, sulfhydryl groups, and labile sulfide in proportions similar to those observed in the Fe–Mo protein of N_2-ase and with $Na_2S_2O_4$ as the reducing agent. Selected results of rate measurements are summarized in Table IX. With acetylene as the substrate, ethylene is produced linearly with time and at rates increasing linearly with the concentration of cluster complex. The catalyst system remains operative for approximately 40 hr and may be regenerated by adding fresh solutions of $Na_2S_2O_4$. Sodium borohydride may also be used and is initially more effective than $Na_2S_2O_4$. However, the cluster complexes are decomposed by $NaBH_4$, yielding black products containing elemental iron.[14] As a consequence, the reaction solutions become rapidly heterogeneous and become less catalytically active. When $Na_2S_2O_4$ is used as the reducing agent, the reaction solutions remain homogeneous for as long as 1 hr and retain the characteristic color of the $Fe_4S_4(SR)_4^{n-}$ anions. The initial rates of C_2H_2 reduction depend on the original oxidation state of the cluster complexes and are greater if n = 4 rather than 2. This result (see Table IX) indicates that *the transfer of electrons from reductant to* $[Fe_4S_4(SR)_4]^{2-}$ *is slow relative to the reaction of*

(14) As evidenced by elemental analysis for Fe, C, H, and S of the decomposition products.

Table IX. Acetylene Reduction with Complex I and Ferredoxin Model Compounds as Catalysts (Turnover Numbers under Various Conditions)

No.	System[b]	Turnover no.[a]
1	$S_2O_4^{2-}$, complex I	0.004
2	No. 1, ATP	0.004
3	No. 1, ferredoxin model (Fe/Mo = 1)	0.65
4	No. 3 (Fe/Mo = 2)	1.4
5	No. 3 (Fe/Mo = 4)	2.3
6	No. 3 (Fe/Mo = 8)	4.9
7	No. 3, ATP (Fe/Mo = 8)	6.2
8	No. 3 (Fe/Mo = 20)	11.3
9	No. 3 (Fe/Mo = 8)[c]	11.0
10	No. 7 (Fe/Mo = 8)[c]	15.2
11	Complex I, ferredoxin model (Fe/Mo = 8)[c]	31.0
12	$S_2O_4^{2-}$, ferredoxin model ([Fe] same as in No. 11)	0
13	BH_4^-, ferredoxin model ([Fe] same as in No. 11)	0
14	BH_4^-, complex I	19
15	No. 14, ATP	35
16	No. 14, ferredoxin model (Fe/Mo = 8)	31[d]
17	No. 15, ferredoxin model (Fe/Mo = 8)	65[d]

[a] Turnover number defined as millimoles of C_2H_2 reduced per mole of Mo per minute. [b] Initial concentrations of complex I: 0.024 mmol in 2 ml of pH 9.6 borate buffer. To this solution, 1.4 ml of a methanolic solution of iron cluster complex containing the calculated amounts was added followed by 0.5 ml of 1 *M* $Na_2S_2O_4$ or 0.5 ml of 1.28 *M* $NaBH_4$ (always freshly prepared), in aqueous pH 9.6 borate buffer; the ATP was added where indicated, 0.5 ml of a 1.28 *M* solution in pH 9.6 borate buffer. All buffers were 0.2 *M*. [c] Here the iron cluster complex was added in its reduced form, *i.e.*, as $[Fe_4S_4(S\text{-}n\text{-}C_3H_7)_4]^{4-}$. In all other cases the oxidized form was used (n = 2). [d] Systems become heterogeneous after the addition of $NaBH_4$.

$[Fe_4S_4(SR)_4]^{4-}$ with the molybdenum catalyst. It is for this reason even possible to employ the tetraanion as a stoichiometric reductant; the rate of acetylene reduction under these conditions is five times that of the rate observed with $[Fe_4S_4(SR)_4]^{2-}$ + $S_2O_4^{2-}$ in comparable systems (Table IX). We find no evidence for the formation of specific stable adducts of the cluster complex anions with the molybdenum catalyst; instead, the rates of acetylene reduction increase linearly with the concentration of cluster over a relatively wide range. In the light of this evidence it is thus possible to formulate the reduction of Mo^{ox} to Mo^{red} as shown in eq 3 and 4. The reduction of Mo^{ox} to Mo^{red} by $NaBH_4$ is

$$Fe_4S_4(SR)_4^{2-} + S_2O_4^{2-} + 2H_2O \xrightarrow{\text{slow}} Fe_4S_4(SR)_4^{4-} + 2SO_3H^- + 2OH^- \quad (3)$$

$$Fe_4S_4(SR)_4^{4-} + Mo^{ox} \xrightarrow[(ATP)]{\text{fast}} \text{[SR, RS, S—Fe, Fe—S, 2e, S, S—Fe—Mo}^{ox}\text{, SR]} \rightarrow Mo^{red} + Fe_4S_4(SR)_4^{2-} \quad (4)$$

accelerated by ATP. The ATP effect is not as pronounced when $S_2O_4^{2-}$ is used in the presence of the sulfur iron cluster complexes, presumably because reaction 3 is too slow.

The system composed of complex I, iron cluster, $Na_2S_2O_4$, and with or without ATP also reduces nitrogen to diimide. At pH 9.6 only traces of ammonia are formed, and the reaction is slower than with $NaBH_4$,

complex I, and ATP. The yield of diimide, as determined by the oxidation of reaction solutions from experiments with $^{30}N_2$ as the substrate, increases during the first 40 hr of reaction and subsequently declines (Figure 4). The shape of the decline curve in Figure 4 suggests that the diimide disproportionates into nitrogen and hydrazine. Analysis of the reaction solutions after 80 hr indeed revealed the presence of 0.8 μmol of N_2H_4, employing the spectrophotometric assay, corresponding to 24% of the amount of diimide after 20 hr of reaction.

D_2–H^+ Exchange Experiments. Nitrogenase holoenzyme appears to promote D_2–H^+ exchange to yield HD more effectively in the presence of nitrogen as the substrate.[15] The same is observed with the model systems containing complex I, ATP, $NaBH_4$, and Fe cocatalyst. The presence of all four components is essential, since little if any exchange occurs in the absence of complex I, ATP, BH_4^-, or Fe cocatalyst. The iron cocatalyst can also be replaced by the ferredoxin model. Nitrogen promotes the D_2–H^+ exchange reaction clearly over that occurring in the absence of reducible substrate. The rate of D_2–H^+ exchange is lowered relative to that under argon in the presence of CO, C_2H_2, CN^-, N_3^-, or CH_2=CHCN, as follows from the data compiled in Figure 5 and Table X. The

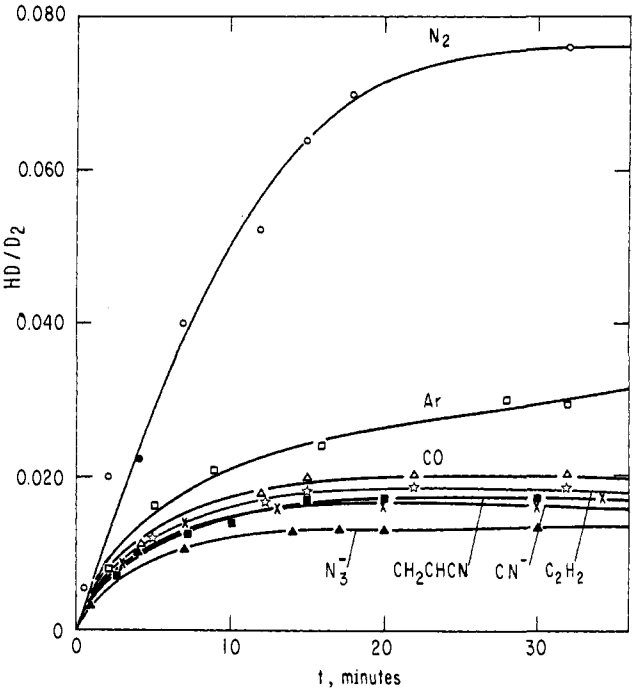


Figure 5. D_2–H^+ exchange by the molybdothiol catalyst system under various conditions and in the presence of substrates or inhibitors. Reaction conditions as given in the legend and footnotes of Table X.

Table X. Relative Rates of D_2–H^+ Exchange under Various Conditions in pH 9.6 Borate Buffer at 25°

No.	System	Substrate [concn]	Relative rate of D_2–H^+ exchange[a]
1	Complex I, ATP, Fe^{2+}, BH_4^-[b]	N_2 [0.1 atm]	100
2	No. 1	Ar [0.1 atm]	37
3	No. 1	C_2H_2 [0.1 atm]	27
4	No. 1	CH_2=CHCN [2.1 m*M*]	26
5	No. 1	CN^- [0.025 m*M* KCN]	25
6	No. 1	N_3^- [0.25 m*M* NaN_3]	17
7	No. 1	CO [0.1 atm]	27
8	Complex I, ATP, $Fe_4S_4(SR)_4^{2-}$, BH_4^-[c]	N_2 [0.1 atm]	86
9	No. 8	Ar [0.1 atm]	43
10	Fe^{2+}–Cys (1:1), ATP, BH_4^-	N_2 [0.1 atm]	34
11	No. 10	Ar [0.1 atm]	34
12	Complex I, ATP, BH_4^-	N_2 [0.1 atm]	8
13	No. 12	Ar [0.1 atm]	11
14	Complex I, BH_4^-	N_2 [0.1 atm]	0.021
15	No. 14	Ar [0.1 atm]	0.036
16	BH_4^-	N_2 [0.1 atm]	(0)
17	BH_4^-	Ar [0.1 atm]	(0)

[a] As determined by the HD–D_2 ratio in the mass spectra of gas samples withdrawn after 20 min of reaction. [b] Reaction solutions contained, where indicated, 0.0075 mmol of complex I, 0.00015 mmol of Fe^{2+} (supplied as $FeSO_4$), and 0.6 mmol of ATP and $NaBH_4$ (initial concentrations), in a total volume of 4 ml, in pH 9.6 borate buffer (0.2 *M*). [c] Ratio of Fe/Mo = 4.

rate of D_2–H^+ exchange is the highest during the first 20 min of reaction, *i.e.*, as long as an excess of reducing agent is present.

Reactions of Diimide. The results in the preceding sections indicate that the reduction of diimide proceeds inefficiently, competing unfavorably with its decomposition. To learn more about the behavior of diimide in our systems we have therefore conducted a number of experiments with diimide generated in solution by the decarboxylation of azodicarboxylate (eq 5). The life-

$$^-O_2CN{=}NCO_2^- \xrightarrow[(H_2O)]{+2H^+} 2CO_2 + N_2H_2 \qquad (5)$$

time of diimide is exceedingly short, both in neutral or mildly alkaline solutions.[10] Decomposition occurs primarily by disproportionation into nitrogen and hydrazine and by decomposition into nitrogen and hydrogen. In pH 7 phosphate buffer at 25° a 0.025 *M* solution of diimide generated according to eq 4 decomposes to about $^1/_3$ into $N_2 + H_2$; the remaining $^2/_3$ of the diimide disproportionates into $N_2H_4 + N_2$, as evidenced by determination of the amount of hydrazine present in the reaction solution.[16] The disproportionation and decomposition of diimide according to eq 6

$$N_2H_2 \begin{cases} \longrightarrow N_2H_4 + N_2 & (6a) \\ \longrightarrow N_2 + H_2 & (6b) \end{cases}$$

is unaffected by the presence of BH_4^-, complex I, ferredoxin model compound, or ATP; in most cases 40–50% of the total amount of diimide is lost through the decomposition according to eq 6b (Table XI). This also means that none of the diimide is reduced to hydrazine, neither by $NaBH_4$ alone nor by the other constituents of our systems. Instead, all the hydrazine is formed by the disproportionation according to eq 6a. However, the subsequent reduction of hydrazine to ammonia is catalyzed by complex I and complex I + ATP. In the presence of the ferredoxin

(15) (a) G. L. Turner and F. J. Bergersen, *Biochem. J.*, **115**, 529 (1969); (b) E. K. Jackson, G. W. Parshall, and R. W. F. Hardy, *J. Biol. Chem.*, **243**, 4952 (1968).

(16) The disproportionation of diimide under the reaction conditions employed yields only traces of ammonia (below 1%), possibly by reaction according to the equation $2N_2H_2 \rightarrow NH_4^+ + N_3^-$ (see ref 10c).

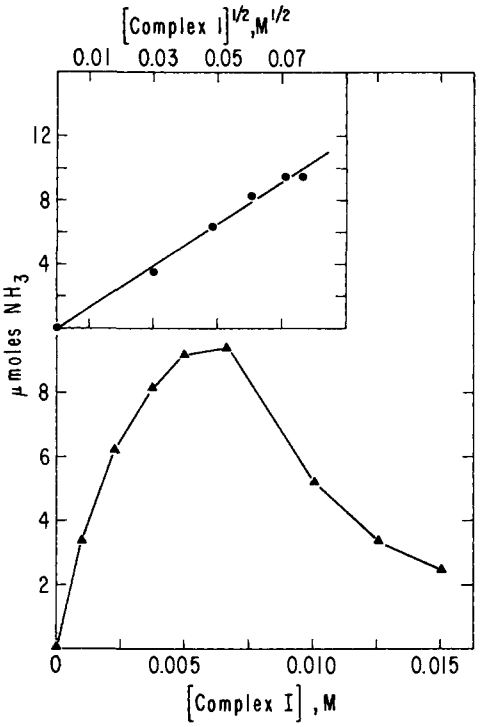


Figure 6. Reduction of hydrazine to ammonia as a function of the concentration of complex I. Reaction solutions in pH 7 phosphate buffer (0.2 *M*) contained 0.006 *M* hydrazine at $t = 0$ and $NaBH_4$ (initial concentration 0.26 *M*); total reaction volume = 3 ml. Ammonia assays were performed in 0.2-ml aliquots withdrawn after 15 min of reaction.

Table XI. Reactions of Diimide (Generated from Azodicarboxylate) under Various Conditions (in pH 7 Phosphate Buffer (0.2 *M*), 25°)[a]

No.	System + N_2H_2	Yields, mol % N_2H_4	Yields, mol % NH_3	Mol of N_2H_2 decomposed
1	$[N_2H_2]_{initial} = 0.025$ *M*	29	0.45	40
2	No. 1, BH_4^-	24	0.74	51
3	No. 2, complex I	23	5.0	44
4	No. 1, complex I	28	1.33	41
5	No. 3, ATP	17	8.3	49
6	No. 2, ferredoxin model[b]	0	21.9	56
7	No. 1, ferredoxin model	32	0	36
8	No. 6, complex I	11	21.4	35
9	No. 6, complex I, ATP	13	12.0	50
10	Na_2MoO_4 (0.30 *M*)	36.7	0.3	26.2
11	Na_2MoO_4 (0.30 *M*), ATP	36.3	0.3	27

[a] Solid dipotassium azodicarboxylate (50 mol) was added to 2 ml of pH 7 buffer solution containing, where indicated, $NaBH_4$ (initial), 0.61 mmol; complex I, 0.024 mmol; ATP (initial), 0.6 mmol; ferredoxin model, 0.012 mmol (of the *n*-propyl derivative). Reaction solutions were allowed to stand for 15 hr prior to ammonia and hydrazine assays. [b] The virtually complete reduction of N_2H_4 to NH_3 in this case is due to the decomposition of the cluster complex under the reaction conditions, giving rise to black deposits containing elemental iron. Iron powder itself reduces hydrazine to ammonia.

model compound all the hydrazine is also reduced to ammonia. However, the reduction in this case is due to the formation of the iron-containing decomposition products of the cluster complex (the reduction of hydrazine to ammonia is catalyzed by elemental iron; see below). Systems containing $NaBH_4$ and ferredoxin model compound reduce the hydrazine for similar reasons even in the absence of complex I.

Reduction of Hydrazine. The reduction of hydrazine to ammonia is catalyzed by complex I, *e.g.*, in pH 9.6 borate or pH 7.0 phosphate buffer, with $NaBH_4$ as the reducing agent. The course of the reaction was followed by determining both ammonia and the remaining hydrazine at various time points during the first 30 min of reaction. The observed nitrogen balance is consistent with the reaction stoichiometry given by eq 7.

$$N_2H_4 + 2e^- + 2H^+ \xrightarrow{\text{[complex I]}} 2NH_3 \qquad (7)$$

The rate of hydrazine reduction increases linearly with $[N_2H_4]_{initial}$ up to the concentration of about 0.02 *M* and increases linearly as a function of [complex I]$^{1/2}$ up to the initial concentration of 0.005 *M* (Figure 6). These results demonstrate that the catalytically active species in *dilute solutions* is *monomeric*, as is the case in the reduction of the other substrates, and that *one molecule* of hydrazine interacts with the catalyst. The K_m value for N_2H_4, determined according to the method of Lineweaver and Burk, is 125 m*M* (in 0.2 *M* pH 9.6 borate buffer at 25°). The reduction of hydrazine is stimulated by ATP as well as by Fe^{2+} cocatalyst but also occurs with iron alone. It is also of interest to note that Cys is not required and that the highest turnover numbers of hydrazine reduction were observed in systems containing only molybdenum (supplied in the form of Na_2MoO_4) and ATP, using $NaBH_4$ as the reducing agent. The addition of Cys actually diminishes the rate of N_2H_4 reduction. Hydrazine evidently has sufficient basicity to act as a ligand for molybdenum and competes with Cys for metal coordination sites. A similar phenomenon (reduction of a substrate in the absence of Cys by Mo_4^{2-}–BH_4^- systems) was observed to a lesser extent with isonitriles.[5] However, in the latter case the addition of Cys still caused a substantial increase of catalytic activity, but this was not observed in the reductions of hydrazine. Hydrazine thus interacts with the molybdenum species to a degree similar to or greater than Cys. The addition of ferredoxin model compounds increases the rate of hydrazine reduction, but this effect is in part caused by the decomposition of the cluster complexes under the strongly reducing conditions. Analysis of the black decomposition products of the reaction of the ferredoxin model compounds with BH_4^- in water–methanol solution indicated the formation of pyrophoric iron (contaminated by solvent- and sulfur-containing products),[14] which was in turn found to catalyze hydrazine reduction themselves. Hydrazine is also readily reduced by iron dust in the presence of BH_4^- (Table XII). The fully reduced cluster complexes, *i.e.*, salts of the anions $[FeS_4(SR)_4]^{4-}$, *do not reduce hydrazine*, however. With $S_2O_4^{2-}$ as the reducing agent, hydrazine reduction by complex I is slow, but it is somewhat accelerated by the addition of the ferredoxin model compound. These results explain why only traces of NH_3 are produced in the reduction of N_2 by complex I, ferredoxin model compound, and $S_2O_4^{2-}$ systems (Figure 5).

Discussion

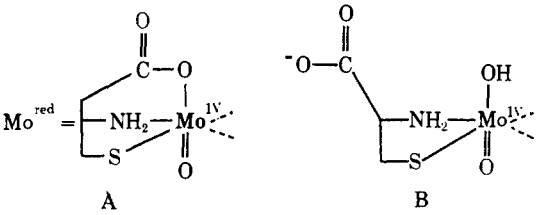
Nitrogen Fixation with Molybdothiol Catalyst Systems. The molybdothiol catalyst systems reduce molecular nitrogen initially to diimide. Under favorable conditions (*e.g.*, in the presence of ATP), diimide is generated in the reaction solution as long as excess re-

Table XII. Reduction of Hydrazine under Various Conditions in pH 9.6 Borate Buffer (0.2 *M*) at 25°

No.	System[a]	$[N_2H_4]_{init}$, μmol	Turnover no.[b]
1	Complex I, Fe^{2+}, ATP, BH_4^-	20	16
2	No. 1, $-Fe^{2+}$	20	14
3	No. 2, −ATP	20	5.0
4	No. 3	50	9.7
5	No. 3	100	23.6
6	No. 3	200	40.2
7	No. 3	400	70.3
8	Na_2MoO_4, BH_4^-	20	35.0
9	No. 8, Cys (1:1)	20	21.0
10	No. 9, ATP	20	47
11	No. 8, ATP	20	62
12	No. 3, $[Fe_4S_4(n\text{-}C_3H_7S)_4]^{2-}$ (Fe/Mo = 4)	20	37
13	No. 12, −complex I	20	21
14	0.048 mmol of Fe dust, BH_4^-	20	18
15	BH_4^- only	20	0
16	$S_2O_4^{2-}$ only	20	0
17	No. 16, complex I	20	0.30
18	No. 17, $[Fe_4S_4(n\text{-}C_3H_7S)_4]^{2-}$	20	2.4
19	No. 18, −complex I	20	1.0
20	$[Fe_4S_4(n\text{-}C_3H_7S)_4]^{4-}$ (0.012 mmol)	20	0
21	Fe^{2+}–Cys (1:1) (0.024 mmol), BH_4^-	20	0

[a] Reaction vials contained, where indicated, complex I, 0.024 mmol; ATP (initial), 0.60 mmol; $NaBH_4$ (initial), 0.61 mmol; $Na_2S_2O_4$ (initial), 0.60 mmol, in a total volume of 4 ml. [b] The turnover number is defined as the millimoles of N_2H_4 reduced per minute per mole of molybdenum (or iron).

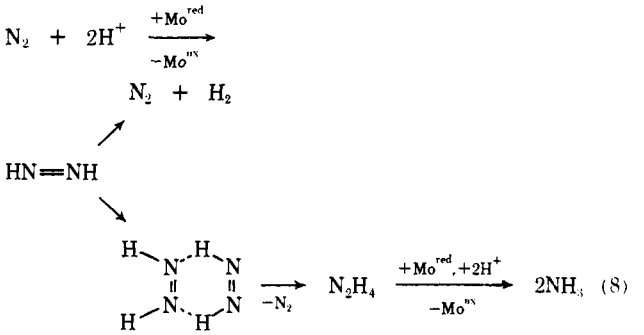
ducing agent is present but subsequently decomposes. In the absence of ATP, reducing power due to BH_4^- is conserved much longer, but the rate of nitrogen reduction is very slow. The small amounts of diimide generated under these conditions in part disproportionate into $N_2 + N_2H_4$; the latter is reduced to ammonia, but since most of the diimide still decomposes into $N_2 + H_2$, the yields of ammonia are low (Figures 1–3). Independent experiments with diimide generated from azodicarboxylate reveal that diimide is apparently not a substrate of the catalyst system. Its disproportionation into hydrazine and nitrogen and the decomposition into nitrogen and hydrogen are largely unaffected by the molybdothiol catalysts, both in the presence or absence of reductant, ATP, or electron-transfer catalysts. Hydrazine, the disproportionation product of diimide, is reduced quite efficiently, in a manner similar to that observed with other substrates. To describe the mechanism of nitrogen reduction to diimide we designate the active reduced form of the catalyst by Mo^{red}. The detailed characterization of this species is difficult, since it is generated only in low stationary concentrations under the reaction conditions and unstable in solution even in the absence of reducible substrates. All previous evidence indicates that it is a 1:1 complex of molybdenum with Cys (or alternate ligands) and that it is *monomeric*, possessing at least two sites for interaction with the substrates.[1-7] We have previously concluded that Mo^{red} contains the metal in the 4+ state of oxidation. This has since been confirmed by polarographic measurements.[17] Possible structures of Mo^{red} are A or B (the coordination of the



A B

carboxylate anion to Mo in A is not essential; A and B may equilibrate).

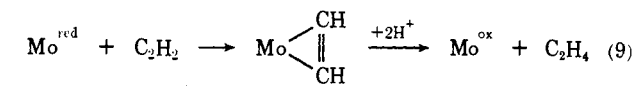
The mechanism of nitrogen reduction may thus be schematically represented as shown in eq 8, where Mo^{ox}



(8)

designates the oxidized (Mo^{6+} or Mo^{5+}) forms of the catalyst. The diimide accumulates in the reaction solutions only under conditions of high electron-transfer efficiency, preferably in the presence of substrate amounts of ATP. In the absence of ATP the amount of diimide generated is lower, but the reaction solutions retain reducing power considerably longer than in the presence of ATP. As a consequence, ammonia is formed through the reduction of the traces of hydrazine resulting from the disproportionation of the diimide. The rate of ammonia production under these conditions is quite slow; the observed turnover number is only 0.1 mmol of N_2 reduced to NH_3 *per hour* per mole of molybdenum. The rate of diimide production in the presence of ATP is considerably greater; the observed turnover numbers, which are uncorrected for the simultaneous decomposition of diimide, are in the order of 3–5 mmol of N_2 reduced to diimide per mole of molybdenum *per minute*, corresponding to approximately 10% of the turnover of acetylene under comparable conditions.

The mechanism of diimide production from nitrogen is considered to be analogous to the mechanism of the reduction of acetylene to ethylene. In the latter reaction, acetylene is reduced probably *via* a symmetrical organomolybdenum intermediate[3] as shown in eq 9.



(9)

The mechanism in eq 9 is supported by the observed stereochemical course of the reduction of acetylene in D_2O (only *cis*-1,2-dideuterioethylene is formed) and the fact that 2-butyne is reduced to *cis*-2-butene.[3]

The mechanism for the reduction of nitrogen to diimide is formulated in analogy to eq 9, involving a side on bonded nitride-type molybdenum complex as the intermediate (eq 10). We have no compel-

(17) (a) M. Ichikawa and S. Meshitsuka, *J. Amer. Chem. Soc.*, **95**, 3411 (1973). (b) The authors of ref 17a have essentially ruled out Mo(III) complexes as possible active forms of our catalyst system. This is in accord with our own findings. The prolonged reduction of complex I with excess BH_4^- in alkaline solution produces dark green species possibly containing Mo(III). These presumably dimeric or polymeric compounds do not react with C_2H_2, or at best only very slowly, and thus cannot be regarded to be the active reduced form of our catalyst.

$$Mo^{red} + N_2 \longrightarrow Mo\begin{matrix}N\\ \|\\ N\end{matrix} \xrightarrow{+2H^+} Mo^{ox} + N_2H_2 \quad (10)$$

ling reason to assume end on bonded nitrogen complexes as the precursors of diimide.[18] In the past, numerous authors have expressed the view that end on bonded nitrogen complexes of transition metals provide models for intermediates in biological nitrogen fixation.[19] It also has been suggested that bimetallic, nitrogen-bridged species of the type M–N_2–M or M–N_2–M′ are involved, where M and M′ are Mo or Fe, respectively. However, the reduction of end on coordinated nitrogen to ammonia or precursors of ammonia often has not been convincingly demonstrated.[20,21] The fact that diimide is not a substrate may be for electronic reasons (the isoelectronic ethylene is also not a substrate) but is primarily a consequence of its extremely short lifetime and thermodynamic instability. The hydrazine formed by the disproportionation of diimide is catalytically reduced; the reduced form of the catalyst interacts with one molecule of hydrazine in this process, as schematically represented in eq 11. Complexes of molybdates in which hy-

$$Mo^{red} + N_2H_4 \longrightarrow Mo(NH_2\text{–}NH_2) \xrightarrow{+H^+} Mo\text{–}NH_2\text{–}\overset{+}{N}H_3 \xrightarrow{+H^+} Mo^{ox} + 2NH_3 \quad (11)$$

drazine functions as an end on bonded ligand are known.[17,22]

Comparison with Nitrogenase. The current hypotheses[19] of the mechanism of biological nitrogen fixation are centered around the assumed formation of an initial adduct or complex of nitrogen with iron and/or molybdenum at the active substrate binding site. Within the most frequently quoted "two-center" hypothesis of nitrogen fixation,[19c,d] nitrogen is assumed to be bound by iron and reduced by molybdenum, whereas the alternate substrates of N_2-ase are bound and reduced by molybdenum alone. Experimental support for the two-center mechanism of nitrogen reduction is essentially indirect and dependent on the interpretation of the results of inhibition experiments. Carbon monoxide inhibits the reduction of N_2 and of all other substrates, while hydrogen appears to inhibit only the reduction of nitrogen. This was interpreted to suggest that nitrogen is reduced at a different site than the remaining substrates. However, it has also been argued that the apparent competitive inhibition of nitrogen fixation by hydrogen is an experimental artifact.[19a] The fact that CO does not inhibit the enzymatic hydrogen evolution has also been quoted in support of the two-center hypothesis, but alternative interpretations of this effect have been advanced.[3] For example, carbon monoxide is likely to interact with the active site in the end on fashion, thus leaving one coordination position accessible for reactions of protons with the medium. The subsequent steps in the reduction of coordinated nitrogen to ammonia have not been clearly defined, but diimide and hydrazine, either free, complexed, or protonated, have been suggested as the precursors of ammonia.[19a] The availability of model systems of the type described in the present paper now permits a number of pertinent conclusions regarding the mechanism of biological nitrogen fixation and the nature of the active site. These will be briefly summarized in the following. Since our model experiments indicate that the reactions of all substrates of N_2-ase are typical of mononuclear molybdothiol complexes, it becomes highly plausible to assume that the active site for substrate binding and reduction in N_2-ase is composed of a single oxomolybdenum ion attached to the apoprotein *via* a cysteine-S^- ion or an equivalent functional group. In the functional holoenzyme the molybdenum site is accessible to molecules or ions of the solvent, to relatively bulky substrates (*e.g.*, crotononitrile), and to the phosphate moieties of ATP. The conversion of oxidized (Mo^{5+} and Mo^{6+}) forms of the molybdothiol catalysts to the active reduced form is a relatively slow process, particularly when $S_2O_4^{2-}$ is employed as the reducing agent, but is effectively accelerated by ferredoxin model compounds of composition $Fe_4S_4(SR)_4^{n-}$ (n = 2 in the oxidized and 4 in the reduced form). Although the structure of the nonheme-iron constituents of the Fe–Mo protein in N_2-ase has not yet been elucidated, it may be expected, in part on the basis of X-ray crystallographic evidence on a Fe_8-non-heme-iron protein,[23] that the active molybdenum site in N_2-ase is surrounded by several (2–4) individual Fe_4S_4(-S-protein)$_4^{n-}$ clusters in close vicinity, to assure the maintenance of the molybdenum active site in the reduced form. In our model systems, ferredoxin-type model compounds have been shown to be effective catalysts of the reduction of Mo^{ox} to Mo^{red}, but these complexes were again found to be reduced relatively slowly by $S_2O_4^{2-}$. Conceivably, the reduction of the nonheme-iron constituents in the Fe–Mo protein of N_2-ase is also slow, requiring the presence of yet another electron-transfer catalyst, which could be the azoferredoxin. The nonheme-iron portions of the Fe–Mo protein may be regarded as storage compartments for electrons. In the molybdothiol model system the reduced ferredoxin cluster model compounds have been successfully employed as stoichiometric reducing agents for the Mo^{red}-catalyzed reduction of acetylene to ethylene. The conversion

(18) The conversion of coordinated nitrogen to yield complexes of diimide has been reported by J. Chatt, G. A. Heath, and R. I. Richards, *J. Chem. Soc., Chem. Commun.*, 1010 (1972). The coordinated diimide could not be reduced further. The complexes studied carry phosphine ligands and contain zero-valent tungsten or molybdenum, thus being distinctly different from our systems.

(19) For a detailed description, discussion, and references see the following books and reviews: (a) J. R. Postgate, Ed., "The Chemistry and Biochemistry of Nitrogen Fixation," Plenum Press, London, 1971; (b) E. N. Mishustin and V. K. Shil'nikova, "Biological Fixation of Atmospheric Nitrogen," Macmillan, London, 1971; (c) R. W. F. Hardy, R. C. Burns, and G. W. Parshall, *Advan. Chem. Ser.*, No. 100, 219 (1971); (d) R. W. F. Hardy and E. Knight, *Progr. Phytochem.*, 1, 407 (1968).

(20) We have also been unable to reproduce the claimed reduction of molecular nitrogen or of nitrogen complexes of molybdenum by sodium naphthalenide in the presence of a sulfur-bridged iron dithiene complex or of ferredoxin model compounds[12,13] described in ref 21 (work with Dr. B. Nahlovsky).

(21) E. E. Van Tamelen, J. A. Gladysz, and J. S. Miller, *J. Amer. Chem. Soc.*, **95**, 1347 (1973); also see *Chem. Eng. News*, 15 (Sept 24, 1973).

(22) (a) For a description of a number of bi- or polynuclear complexes of hydrazine and molybdates, see ref 22b. The demonstrated dependence of the rate of reduction on [complex I]$^{1/2}$ (see Figure 7 above) rules out binuclear molybdate–hydrazine complexes as catalytic intermediates in dilute solution under our reaction conditions. This does not eliminate the possibility that binuclear or polynuclear molybdates may possess catalytic activity under different experimental conditions. (b) P. C. H. Mitchell and R. D. Scarle, *Nature (London)*, **240**, 417 (1972).

(23) L. C. Sieker, E. Adman, and L. H. Jensen, *Nature (London)*, **235**, 40 (1972).

of oxidized forms of the Mo–Cys catalysts (Mo^{ox}) to Mo^{red} is stimulated by ATP and other nucleoside phosphates. We have previously shown that the concurrent hydrolysis of ATP to ADP and P_i in the model systems is not coupled to the catalyst activation step. This may also be the case in the enzyme. The effect of ATP in N_2-ase is undoubtedly potentiated by the presence of specific ATP binding sites in the apoprotein, however, which are absent in the current model systems.

The first substrate-dependent step in biological nitrogen fixation may now be described as the reaction of N_2 with the reduced molybdenum active site, leading to the formation of a side on bonded nitride intermediate whose subsequent hydrolysis gives rise to diimide. The diimide presumably remains in the vicinity of the active site and could perhaps be protonated, but its subsequent reactions must be viewed under the aspect of its short lifetime and extreme reactivity. It is possible that the diimide is reduced to hydrazine under enzymatic conditions, but all available enzymological evidence would not be incompatible with the assumption that diimide, once formed, *disproportionates* to nitrogen and hydrazine in the vicinity of the active site. Some of the diimide may also decompose into nitrogen and hydrogen. In fact, the observed catalysis of H_2–H^+ exchange reactions by N_2-ase or in the model systems in the presence of nitrogen as the substrate may well be associated with the partial decomposition of diimide under the reaction conditions. The subsequent reduction of hydrazine to ammonia could occur at the molybdenum active site and has been adequately accomplished under nonenzymatic conditions. Hydrazine is assimilated by growing cultures of nitrogen-fixing organisms, but its reduction by N_2-ase *in vitro* has not yet been accomplished. The molybdothiol model systems reduce all substrates of N_2-ase at rates approaching 0.01% of the enzyme on a per-molybdenum basis.[7] The lower efficiency of the model catalysts is primarily due to the slower rate of electron transfer to molybdenum and the low stationary concentration of active reduced catalyst under the currently employed reaction conditions. The turnover numbers, obtained from conservative estimates, may probably be increased by further variations of the reaction conditions.[23a] However, it also appears that the model systems exhibit a generally lower affinity for the substrates of N_2-ase than the enzyme, as evidenced by the higher nonenzymatic K_m values.[7] This may be due to the low molecular weight of the model catalysts or by differences in the electron density or the effective reduction potentials between the enzyme-bound molybdenum and the nonenzymatic models.

Concluding Remarks. The consequential application of models in the study of metalloenzymes provides a new means for the elucidation of the mechanism of complex biological processes and establishes primary functional and chemical affinity relationships between the reactive components of the natural systems. This approach has been successful for the specific case of biological nitrogen fixation and led to the formulation of an empirically supported theory of the mechanism of nitrogenase action. The acceptance of conclusions derived by this method depends ultimately on the degree of recognition of the importance of models and analogies in science. The validity of this approach has been adequately treated and justified in the more recent philosophical literature.[24]

Experimental Section

Reagents and Chemicals. Labeled nitrogen, $^{30}N_2$ (99+%), was obtained from two commercial sources.[25] The purity and sources of all other reagents was the same as outlined in ref 7 or was reagent grade or better. Complex I and the 4:1 molybdenum–glutathione complex were synthesized according to published procedures.[11] For the synthesis of the ferredoxin model compounds a general method is outlined below.

Nitrogen Fixation Experiments with the Standard N_2-ase Model System. Numerical details for the conditions used in individual experimental series of nitrogen fixation runs are given in the legends of the figures and the tables. A typical procedure is described in the following. Screw-capped, rubber septum vials (obtained from Precision Sampling Corp., Baton Rouge, La. 70815, Catalogue No. 630063) were first evacuated and then filled with 1 atm of 99+ atom % $^{30}N_2$. Subsequently, 3 ml of freshly prepared solutions of complex I (*e.g.*, 0.008 *M*) in pH 9.6 0.2 *F* borate or phosphate buffer was injected, followed by 0.5 ml of freshly prepared 1.33 *F* $NaBH_4$ in pH 9.6 buffer. In the experiments without added ATP, the reaction vials were allowed to stand without agitation for between 20 and 200 hr. For analysis, the solutions were first carefully acidified by adding 0.2 ml of 12 *N* HCl and quantitatively transferred into one of the limbs of a Rittenberg apparatus. A freshly prepared solution (usually 1.5 ml) of alkaline hypobromite was placed into the second limb of the Rittenberg apparatus. The following experimental steps were exactly as described in ref 9; the N_2 was collected by means of a Toepler pump into a sampling vessel, which was then transferred to the inlet of the mass spectrograph, a LKB 9000 model, for analysis. Most measurements were performed at gain 6; all data are corrected for background (the required corrections were insignificant relative to the observed peak heights); the $^{29}N_2$ peaks are all corrected, by subtracting the calculated $^{29}N_2$ peak height from the observed, assuming the natural abundance of $^{29}N_2$ to be 0.74%.

In the experiments with ATP, a freshly prepared 1.2 *M* solution in pH 9.6 buffer (usually 0.5 ml) was injected after the addition of the $NaBH_4$. At the times indicated, the reaction solutions were stopped by the addition of 0.2 ml of 12 *N* HCl and either analyzed as described above or were made alkaline again by the addition of aqueous NaOH and then distilled. The distillates were oxidized with hypobromite as usual. As mentioned in the Results, some unlabeled NH_4Cl was frequently added to assure the quantitative conversion of $^{15}NH_3$ into $^{29}N_2$ on hypobromite oxidation.

The colorimetric assays for ammonia and hydrazine are described in ref 26 and 27. The hydrazine assay was modified by extracting the colored hydrazone into CH_2Cl_2 followed by measurement of its absorbance at 490 mμ. Since ferric ion interferes with the ammonia assay, excess NaF was added to convert it into FeF_6^{3-}, in all cases where iron was present in the reaction solutions.

D_2–H^+ Exchange Studies. One limb of a Rittenberg apparatus was filled with 3.0 ml of a freshly prepared 0.025 *M* solution of complex I, followed by 0.5 ml of 1.2 *M* ATP and 0.5 ml of a 0.0034 *M* aqueous solution of $FeSO_4 \cdot 7H_2O$. Into the other limb of the Rittenberg apparatus, 0.5 ml of a freshly prepared 1.33 *M* $NaBH_4$ solution was added; all reagent solutions were prepared in 0.2 *F* borate buffer of pH 9.6. Both limbs of the Rittenberg apparatus were frozen with liquid nitrogen and evacuated on a high-vacuum system. The evacuated Rittenberg apparatus was subsequently filled with 10 cc of gaseous D_2, followed by 15 cc of N_2 or C_2H_2.

(23a) NOTE ADDED IN PROOF. With acetylene as the substrate, turnover numbers approaching 1% of reported values for N_2-ase were recently obtained with our complex I–$NaBH_4$ model system by *lowering* the catalyst concentration to 0.5–2 μ*M*. The smaller turnover numbers observed at higher concentrations of catalyst are attributed primarily to the increased formation of inactive binuclear molybdenum complexes.

(24) See M. B. Hesse, "Models and Analogies in Science," University of Notre Dame Press, Notre Dame, Ind., 1966.

(25) The British Oxygen Company Ltd. or Monsanto Research Corp. (Miamisburg, Ohio).

(26) J. Kruse and M. G. Mellon, *J. Water Pollut. Contr. Fed.*, **24**, 1098 (1952).

(27) G. W. Watt and J. D. Chrisp, *Anal. Chem.*, **24**, 2006 (1952).

After thawing, the reaction solutions were mixed and gas samples were withdrawn at various intervals for the mass spectrographic determination of the HD–D_2 ratios. The results of these and other experiments are summarized in Table X.

Synthesis of Ferredoxin Model Compounds.[12,13] Following the initial report on complexes containing the anions $[Fe_4S_4(SR)_4]^{2-}$, a number of representatives of this class of compounds were synthesized. Initially, the complexes were prepared with R = ethyl, but these were found to undergo slow decomposition under reducing conditions, giving rise to ethylene and ethane derived from the ethylmercaptide residues. In the presence of complex I and reducible substrate, this decomposition is inhibited, but the independent formation of ethylene and ethane from the electron-transfer catalyst was sufficiently disturbing for us not to use this complex anion for our experiments. Instead, we prepared the corresponding anion with R = *n*-propyl, which is more stable under reducing conditions. Although traces of propylene and propane are still formed, these hydrocarbons do not interfere with the gas chromatographic assay for C_2 hydrocarbons in acetylene reduction experiments. The ferredoxin model compounds were either employed in the form of the bis(tetra-*n*-butylammonium) salts or as the dilithium derivatives. The former are less soluble and readily obtained in crystalline form; the latter are more difficult to obtain in substance and were generated *in situ*.

(a) **Synthesis of** $[N(n\text{-}C_4H_9)_4^+]_2[Fe_4S_4(S\text{-}n\text{-}C_3H_7)_4]^{2-}$. Anhydrous ferric chloride, 5.2 g (32 mmol), was dissolved in 20 ml of anhydrous methanol. To this solution a methanolic solution of lithium *n*-propylmercaptide was added. The latter was prepared by adding 3.65 g of $LiOCH_3$ to 40 ml of a solution of 8.6 ml of freshly distilled *n*-propylmercaptan in 40 ml of anhydrous CH_3OH. The reaction of ferric chloride with the lithium mercaptide causes the precipitation of dark green iron *n*-propylmercaptides. These were not isolated but instead reacted further with 1.46 g of solid, anhydrous Li_2S (available from Alfa Inorganics). This produces a dark, bronze-colored homogeneous solution. The complex ion was precipitated by the addition of 9.2 g of $N(n\text{-}C_4H_9)_4Br$, dissolved in 40 ml of anhydrous methanol. The black crystalline salt was filtered off, washed with methanol, and dried at room temperature under reduced pressure.

Anal. Calcd for $N(n\text{-}C_4H_9)_4^+]_2[Fe_4S_4(S\text{-}n\text{-}C_3H_7)_4]^{2-}$: C, 45.37; H, 8.65; S, 22.00 Fe, 19.18. Found: C, 45.22; H, 8.45; S, 23.0; Fe, 19.9.

For the experiments with the lithium salt of the cluster anion, the solution resulting from the addition of Li_2S to the suspension of iron propylmercaptide was employed without further purification. The Fe(II) form of the cluster complex was generated by direct synthesis as described above but with methanolic solutions of $FeCl_2$ instead of $FeCl_3$. The $FeCl_2$ solution was prepared by dissolving 4.06 g of anhydrous ferric chloride in 20 ml of anhydrous methanol. The resulting solution was converted to the ferrous salt by the addition of iron dust and was used as described above, after removal of excess elemental iron.

(b) **Acetylene Reduction Experiments.** Acetylene-filled (1 atm) reaction vials of 25-ml volume were filled with 1.5 ml of an aqueous solution of 0.106 *M* complex I in pH 9.6 borate buffer (0.2 *M*). This was followed by 1.4 ml of the methanolic solution of $Li_2Fe_4S_4(S\text{-}n\text{-}C_3H_7)_4$ as obtained by the above procedure or by adding the corresponding amount of the tetrabutylammonium salt in methanol. Finally, 0.5 ml of freshly prepared, 1.2 *M* pH 9.6 buffered solutions of the reducing agents were added (either $Na_2S_2O_4$ or $NaBH_4$), followed by 0.5 ml of a freshly prepared 1.2 *M* solution of ATP, where indicated (see Table IX). Gas samples of 0.1 ml were periodically withdrawn for product analysis by glpc, using a Hewlett-Packard Series 700 laboratory instrument equipped with a phenyl isocyanate–Porasil 80–100 mesh column.

(c) **Nitrogen Reduction Experiments.** The experiments with nitrogen as the substrate were performed under conditions essentially identical with those described in b, except that the vials where initially filled with 99+% $^{30}N_2$ at 1 atm. Product analysis was carried out by the standard hypobromite oxidation method as described above of the degassed and previously acidified reaction solutions or aliquots thereof.

Experiments with Chemically Generated Diimide. Solid dipotassium azodicarboxylate, in portions of 10.0 mg, was added to pH 9.6 borate buffered solutions of the reaction components listed in Table XI. After 15 hr of reaction at 25°, aliquots of 0.4 ml were withdrawn for ammonia and hydrazine analysis by the spectrophotometric methods.

Hydrazine Reduction. The experiments in Table XII were performed in reaction vials containing the amounts of reagents indicated in the footnotes. Ammonia and hydrazine were assayed spectrophotometrically.

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Electron-Carbon Couplings of Aryl Nitronyl Nitroxide Radicals

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Abstract: We have taken ^{13}C nmr spectra of a group of aryl nitronyl nitroxide radicals to determine the sign and magnitude of the electron–carbon coupling constants. Lines were observed from the aliphatic carbons on the nitronyl nitroxide ring and from all of the aromatic carbon atoms except the bridgehead carbon. The ^{13}C coupling constants of the aromatic carbon atoms are used to estimate spin polarization parameters. The ortho carbon couplings are found to be larger than predicted. The magnitude of these coupling constants can be explained by either an unexpectedly large spin density at the bridgehead carbon or by a long range interaction with spin in the nitronyl nitroxide ring. The coupling constants of the saturated carbons on the nitronyl nitroxide ring are discussed in terms of a mechanism involving polarization of the saturated bonds by spin at the nitroxide group.

The low natural abundance of ^{13}C generally makes electron spin resonance measurements of electron–carbon hyperfine coupling constants difficult or impossible. Carbon couplings which have been measured have come from compounds in which the carbon hyperfine lines either lie outside of the main part of the esr spectrum or are spaced between other hyperfine

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