CD results on *C. magister* hemocyanin. The valuable assistance of Dr. Jeffrey Freedman during many phases of this work is deeply appreciated.

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The Chemical Evolution of a Nitrogenase Model. XI. Reduction of Molecular Nitrogen in Molybdocyanide Systems

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Abstract: Using $^{30}N_2$-enriched nitrogen as the substrate, the reduction of molecular nitrogen to ammonia is demonstrated in systems containing the complex anion $[Mo(O)(H_2O)(CN)_4]^{2-}$ and substrate amounts of ATP and $NaBH_4$. As in the molybdothiol model systems of nitrogenase, diimide, N_2H_2, is shown to be the first product of nitrogen reduction. The diimide subsequently disproportionates and in part decomposes to hydrazine, N_2 and H_2. Hydrazine is in turn reduced to ammonia; independent experiments show that this reaction is also catalyzed by molybdocyanide species and stimulated by ATP. The addition of ferredoxin model compounds as electron transfer catalysts enhances nitrogen reduction; CO, C_2H_2, CN^-, and O_2 cause partial or complete inhibition. The reduction of molecular nitrogen also occurs in systems containing $MoO_4{}^{2-}$ and CN^- in the molar ratios of 1:1 or 1:2, in the presence of $NaBH_4$ and ATP.

In part X of this series[1] we demonstrated that the well-defined[2] complex $K_2[Mo(O)(H_2O)(CN)_4]$ may serve as a model for a substrate-catalyst complex in the simulation of the reduction of CN^- by nitrogenase (N_2-ase).[3,4] The coordinated CN^- in this anionic Mo^{4+} complex is reduced to NH_3, CH_4, C_2H_4, C_2H_6, and traces of CH_3NH_2 by reducing agents such as $NaBH_4$ or reduced ferredoxin model compounds. During the reaction, reactive cyanide complexes of oxomolybdate with free coordination sites are formed which catalyze the reduction of other substrates of N_2-ase, including molecular nitrogen. In part VII of this series it was shown that the reduction of N_2 in molybdothiol model systems of N_2-ase pro-

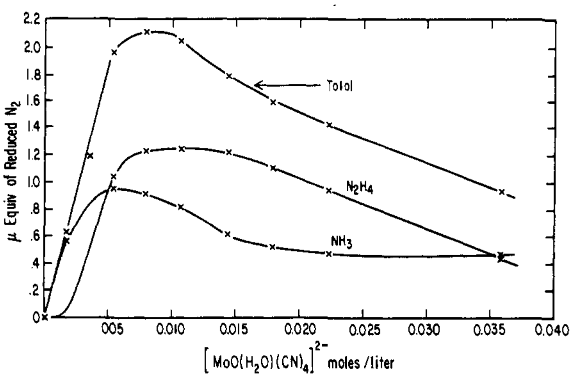
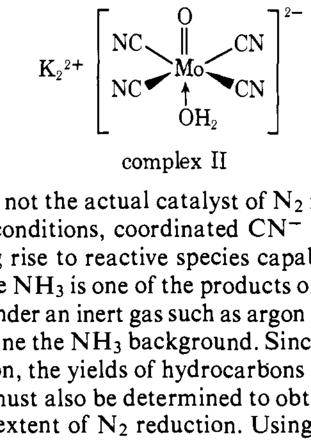


Figure 1. Reduction of molecular nitrogen as a function of the concentration of complex II. Reaction solutions contained in a total volume of 4 ml: $NaBH_4$, 0.08 M; ATP, 0.04 M (initial concentrations). Initial pH adjusted to 7.5 (0.2 M borate buffer). Gas phase nitrogen at 1 atm, enriched with 10 vol % N_2. Reaction time was 6 h; temperature was 20 °C. Reactions were stopped with 1 ml of 6 N HCl.

ceeds via the intermediates diimide, N_2H_2, and hydrazine, N_2H_4.[5] In the present paper we demonstrate that the reduction of N_2 with molybdocyanide catalysts occurs by a similar mechanism.

Results

Reduction of N_2 with Molybdocyanides as Catalysts. Experimental Design. All experiments to be described were performed in homogeneous, protic reaction systems, containing $NaBH_4$ as the reductant, usually in the presence of substrate amounts of ATP and with solutions of the salt $K_2[Mo(O)(H_2O)(CN)_4]$ as the catalyst precursor. The latter will be designated "complex II" to simplify the notation and to differentiate it from "complex I", the binuclear Mo^{5+} complex of L-(+)-cysteine, employed in most of our previous work.



complex II

Complex II is not the actual catalyst of N_2 reduction. Under the reaction conditions, coordinated CN^- is reductively removed, giving rise to reactive species capable of interacting with N_2. Since NH_3 is one of the products of CN^- reduction, control runs under an inert gas such as argon have to be carried out to determine the NH_3 background. Since N_2 also inhibits CN^- reduction, the yields of hydrocarbons generated during the reaction must also be determined to obtain accurate estimates of the extent of N_2 reduction. Using modified colorimetric assays (see Experimental Section), nitrogen fixation to ammonia and hydrazine could be directly demonstrated even without employing isotopically labeled substrate. However, in the present study, we performed most of the experiments with $^{30}N_2$-enriched N_2. This offered several advantages. For example, if $^{15}NH_3$ is formed together with excess amounts of $^{14}NH_3$, oxidation of the reaction solutions by OBr^- gives rise to the formation of $^{29}N_2$, the yields of which can be readily determined by mass spectrographic analysis after correction for natural abundance of ^{15}N. Precursors of NH_3 such as N_2H_4 and N_2H_2, or, if present, N_2 complexes of the catalyst, will give rise to $^{30}N_2$ on oxidation with OBr^-. It is thus possible to detect intermediates and products of the reactions of N_2 simultaneously and to prove the formation of NH_3 unambiguously.

Reduction of N_2 in Systems Containing Complex II. Systematic experiments revealed that the reduction of N_2 with complex II and $NaBH_4$ proceeds optimally under conditions which are also optimal for the reduction of coordinated CN^-. In Figure 1, the total yields of N_2 fixed are plotted as a function of the initial concentration of complex II. It may be seen that the optimal catalyst concentration is between 0.005 and 0.010 M and that higher concentrations of complex II are inhibitory, just as in the reduction of coordinated CN^-.[1] In Figure 2, the dependence on the initial ATP concentration is shown. Little N_2 is fixed in the absence of ATP, while maximum fixation occurs at $[ATP]_{initial} = 0.04$ M. The results of a time-dependent study are represented graphically in Figure 3. At 25 °C, the highest yields of ^{15}N-containing products are found in the solutions after 5–10 min of reaction. Since the reduction of CN^- proceeds continuously with time, it is obvious that the reduction of N_2 has some anomalous features. A similar time dependence of the yield of labeled nitrogen product(s) was observed in the study of nitrogen reduction with molybdothiol catalysts and was traced back to the intermediate formation of diimide.[5-7] The temperature dependence of N_2 fixation is also anomalous; i.e., more labeled nitrogen accumulates in the reaction solutions in experiments carried out at 8–10 °C than at higher temperatures (Figure 4). This behavior is again suggestive of the formation of reactive intermediates of N_2 reduction and parallels the previous findings with molybdothiol catalysts. In Table I numerical results of typical experiments are given which demonstrate the essentiality of all components (molybdenum catalyst, BH_4^-, and ATP) for N_2 fixation in these functional model systems of N_2-ase, as well as the effect of a ferredoxin model compound[8] as electron transfer catalyst. The results are in qualitative agreement with those reported in ref 1; the yield differences are due to the different reaction conditions employed.

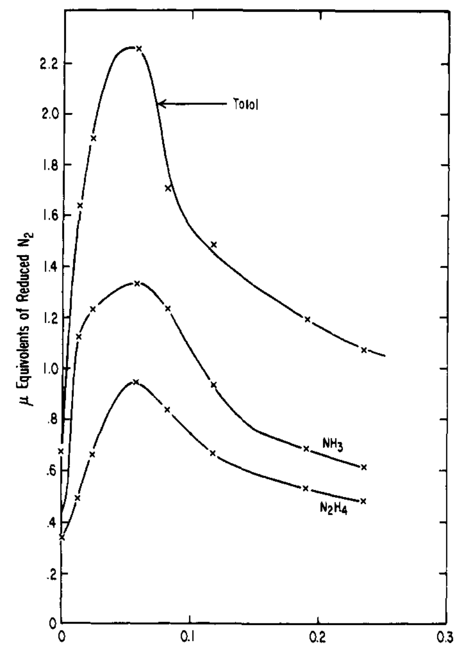


Figure 2. Dependence of nitrogen reduction on the ATP concentration. Reaction solutions contained in a total volume of 4 ml: complex II, 0.0054 M; $NaBH_4$, 0.08 M (initial concentrations), and ATP as indicated. Gas phase was nitrogen, enriched with 10 vol % $^{30}N_2$ at 1 atm. Reaction time was 6 h; reaction temperature was 20 °C. Reactions were stopped with 1 ml of 6 N HCl.

Identification of Diimide, Hydrazine, and Ammonia. To demonstrate the intermediate formation of diimide, experiments were performed in which reaction solutions were quenched by addition of solutions of sodium succinate and fumarate, respectively. Table II shows that the addition of succinate has no effect on the total yield of $^{30}N_2$, which is re-

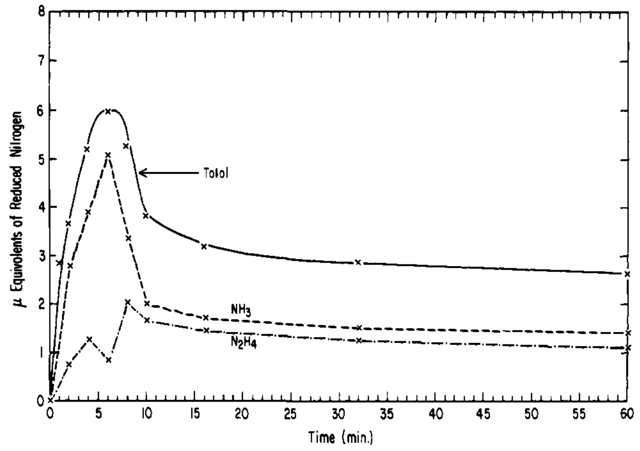


Figure 3. Dependence of nitrogen fixation on the reaction time. Reaction solutions contained in a total volume of 4.0 ml: complex II, 0.0054 M; ATP, 0.04 M; $NaBH_4$, 0.08 M (initial concentrations), in pH 7.5 0.2 M borate buffer. Gas phase was nitrogen at 1 atm, enriched with 10 vol % $^{30}N_2$. Temperature was 20 °C. Reactions were stopped with 1 ml of 6 N HCl.

Table I. Yields of NH_3 and N_2H_4 from Molecular N_2 in Molybdocyanide Model Systems of Nitrogenase and Effects of Omission of System Components (reaction temperature, 10 °C; substrate, $^{30}N_2$-enriched N_2 at 1 atm)[a]

No.	System	Yields (μ-atom equiv) NH_3	N_2H_4	Total	Relative yields
1	Complete	6.30	0.63	6.93	100
2	-Ferredoxin model compound	3.10	0.92	4.02	58
3	2, -ATP	0.77	0.45	1.22	18
4	2, -BH_4^-	0	0	0	0
5	2, -complex II	0.1[b]	0.1[b]	0.2[b]	(0)
6	MoO_4^{2-}, CN^- (1:1), ATP, BH_4^-	1.75	0.20	1.95	28
7	6, -CN^-	0.30	0.15	0.45	6.5
8	6, -ATP	0.21	0.10[b]	0.31	(4.5)
9	6, -BH_4	0	0	0	0
10	6, -MoO_4^{2-}	0	0	0	0

[a] At approximately optimal catalyst, ATP and BH_4^- concentrations. The complete system contained the components in the following initial concentrations: complex II, 0.0054 M; ATP, 0.04 M, BH_4^-, 0.08 M. Initial pH: 7.5 (0.2 M borate). Total reaction volume: 4.0 ml. The electron transfer catalyst (ferredoxin model compound) was added in the form of the salt $[N(n\text{-}C_4H_9)_4^+]_2[Fe_4S_4(S\text{-}n\text{-}C_3H_7)_4]^{2-}$ at the initial concentration of 0.0054 M. In experiment no. 6, the concentrations of MoO_4^{2-} and CN^- were 0.0054 M. Reaction solutions were quenched with 1 ml of 6 N HCl after 6 h of reaction. The vacuum-degassed reaction solutions were subjected to OBr^- oxidation, the N_2 released was collected and analyzed for $^{28}N_2$, $^{29}N_2$, and $^{30}N_2$. Yields of products were determined from the observed mass spectrographic peak heights; $^{29}N_2$ was corrected for natural abundance from the observed yield of $^{28}N_2$, assuming the natural abundance of ^{15}N to be 0.74%. The substrate N_2 contained 10 vol % $^{30}N_2$; the yields are the average from several determinations. The yields of NH_3 were determined from the observed yields of corrected $^{29}N_2$, those of N_2H_4 (N_2H_2) from the observed yields of $^{30}N_2$. [b] Probably background.

leased upon subsequent hypobromite oxidation. With fumarate, however, the yield of $^{30}N_2$ drops to below 50% of the original amount (Table II). The observed loss of $^{30}N_2$ upon addition of fumarate is attributed to its reaction with N_2H_2 according to eq 1:

$$N_2H_2 + {}^-O_2CCH{=}CHCO_2^- \rightarrow N_2 + {}^-O_2CCH_2CH_2CO_2^- \quad (1)$$

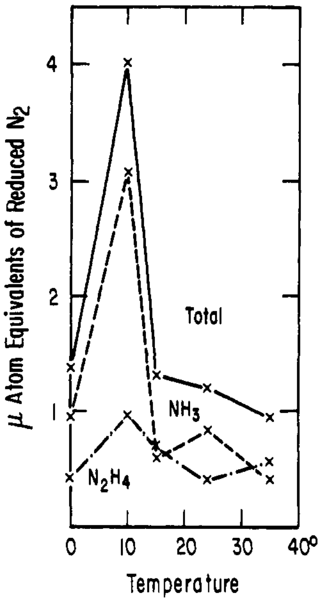


Figure 4. Temperature dependence of nitrogen fixation. Reaction solutions contained in a total volume of 4.0 ml: complex II, 0.0054 M; ATP, 0.04 M; $NaBH_4$, 0.08 M (initial concentrations), in 0.2 M pH 7.5 borate buffer. Gas phase was nitrogen at 1 atm, enriched with 10 vol % $^{30}N_2$. Yields were measured after 6 h of reaction. Reactions were stopped with 1 ml of 6 N HCl.

Table II. Effect of Addition of Sodium Succinate and Fumarate on the Yields of Labeled Nitrogen in the Reaction Solutions

No.	Conditions	μ-Atom equiv after OBr^- oxidation N_2H_4	NH_3	Total
1	Same as in expt no. 2, Table I, but at 20 °C, acid quenched after 40 min of reaction	0.70	1.50	2.20
2	Same as in 1, added 0.5 ml of 0.2 M succinate after 10 min of reaction	0.70	1.50	2.20
3	Same as in 1, added 0.5 ml of 0.2 M fumarate after 10 min of reaction	0.31	0.58	0.86

Table III. Recovery of Labeled Nitrogen after Distillation of the Reaction Solutions

No.	μ-Atom equiv before (after) distillation[a] $^{29}N_2$	$^{30}N_2$	$^{15}N_{total}$	% recovery
1	0.60 (1.20)	2.08 (0.72)	2.68 (1.92)	71.6
2	0.80 (1.04)	1.76 (0.80)	2.56 (1.84)	71.9
3	1.16 (1.32)	1.20 (0.80)	2.36 (2.12)	89.8
– Av	0.84 (1.16)	1.68 (0.76)	2.52 (1.96)	77.8

[a] Analysis of acidified aliquots. Reaction solutions were: 0.0107 M in complex II, 0.04 M in ATP, 0.265 M in $NaBH_4$ (initial concentrations). Total solution volume: 4.0 ml. Initial pH: 7.5 (0.2 M borate). Reaction time: 40 min. Temperature: 25 °C. Reaction was terminated by the addition of 2 ml of 1 N HCl. Solutions were made alkaline prior to distillation at normal pressure under argon.

In other experiments, aliquots of reaction solutions were analyzed for labeled nitrogen before and after distillation. The results in Table III demonstrate that the total recovery of labeled nitrogen is in the order of 70-90%. During the distillation of the reaction solutions, losses of N_2H_4 containing the original N-N bond occur, due partly to its reduction to NH_3 and partly to its decomposition into $^{30}N_2$. The reaction solutions still possess residual reducing power due to the presence of reduced molybdenum species, and these evidently convert hydrazine to ammonia. Similar losses of N_2H_4 on distillation under the same conditions were also observed if N_2H_4 was added to solutions containing $NaBH_4$, ATP, and complex II after consumption of $NaBH_4$ was essentially complete. Table III shows that on the average 35.7 equiv % of the total amount of $^{30}N_2$

Table IV. Effect of Acid-Quenching on the Yields of $^{29}N_2$ and $^{30}N_2$ after Hypobromite Oxidation

No.	Reaction time	Conditions[a]	Yields (μ-atom equiv) $^{29}N_2$	$^{30}N_2$	$^{15}N_{total}$
1	40 min	Unquenched	0.85	1.20	2.05
2	40 min	Quenched	1.50	0.70	2.20
3	18 h	Unquenched	0.70	1.04	1.74
4	18 h	Quenched	0.70	0.27	0.97

[a] Concentrations of components as in experiment no. 2, Table I, reaction temperature 20 °C. Where indicated, solutions were quenched with 1 ml of 6 N HCl.

Table V. Nitrogen Reduction with Complex II in the Presence of the Anion $[Fe_4S_4(SR)_4]^{2-}$ (R = n-C_3H_7)[b]

No.	Mo:Fe ratio	Yields after OBr^- oxidation (μ-Atom equiv)[a] NH_3	N_2H_4	Total
1	1:0	3.10	0.95	4.05
2	1:1	2.85	1.40	4.25
3	1:4	6.30	0.61	6.91

[a] Reaction solutions were quenched with 1 ml of 6 N HCl before OBr^- oxidation. [b] Reaction temperature: 10 °C; yields were measured after 6 h of reaction. Concentration of complex II, ATP, and BH_4^- as in experiment no. 2, Table I.

Table VI. Inhibition of Nitrogen Reduction

Inhibitor	Yields, μ-atom equiv[a] NH_3	N_2H_4	Total	% inhibition
None	3.22	0.95	4.17	(0)
CO, 0.5 atm	0.29	0.01	0.30	93.8
CN^-, 0.02 M	0.35	0.03	0.38	90.3
C_2H_2, 0.5 atm	0.33	0.02	0.35	89.8
O_2, 0.5 atm	0.29	0.03	0.32	91.8

[a] Concentrations of system components as in experiment no. 2, Table I, reaction temperature 10 °C. Yields were measured after 6 h of reaction; solutions were quenched with 0.5 ml of 6 N HCl prior to hypobromite oxidation.

Table VII. Effects of ATP, ADP, AMP, and of Four Different Acid Anions on N_2 Reduction Catalyzed by Complex II and the Inhibition of CN^- Reduction by Nitrogen. Concentrations of Reactants as in Expt 3, Table I

No.	Component added[a]	Yield of NH_3[b] (μmol)	% inhibition of CN^- Reduction by N_2[c]
1	ATP	2.2–2.7[d]	30–80[d]
2	Hydrolyzed ATP	0	0–10
3	ADP	Trace	0
4	AMP	0.2	ca. 2
5	PO_4H^{2-}	0.6	0
6	SO_4^{2-}	0.07	0
7	Cl^-	Trace	0
8	ClO_4^-	0	0

[a] All initial concentrations 0.04 M. [b] Determined colorimetrically. [c] As determined from the hydrocarbon production. [d] Variation of NH_3 yields and the percent of N_2 inhibition of CN^- reduction depends on reaction conditions and the degree of ATP hydrolysis.

is lost during distillation, 19% is reduced to NH_3 (i.e., appears as $^{29}N_2$ after OBr^- oxidation), and 45.2% is recovered as N_2H_4. The presence of N_2H_4 in the distillates was confirmed by colorimetric assay.

Effect of Quenching. The relative yields of $^{29}N_2$ and $^{30}N_2$ released upon OBr^- oxidation of the reaction solutions depend on reaction time and method of workup. After short reaction times (5-15 min), the yields of $^{30}N_2$ are as a rule higher if OBr^- is added to the reaction solutions without prior quenching. If the reactions are first stopped by adding acid (e.g., 1 ml of 6 N HCl), the yields of $^{30}N_2$ are lower, while those of $^{29}N_2$ increase. This is attributed to the presence of unreacted $NaBH_4$, which reduces some of the N_2H_4 during the acid addition to NH_3. After long reaction times (18 h), acid-quenching still causes a loss of $^{30}N_2$, but does not also increase the yield of $^{29}N_2$. Under these conditions the $NaBH_4$ is essentially consumed; during acidification some of the N_2H_4 present evidently is oxidized to N_2 (Table IV). The results in Figure 3 indicate that the yields of NH_3 and N_2H_4 change as a function of time. The highest yields of NH_3 are observed after short reaction times (2-10 min), i.e., under conditions where unreacted $NaBH_4$ is still present. Since the reaction solutions were acid quenched, these data also indicate that the N_2H_4 in the solutions is reduced to NH_3 during the quenching process, as long as $NaBH_4$ is still available. As $NaBH_4$ is being consumed, less NH_3 is produced upon acidification of the reaction solutions.

Reduction of Hydrazine. Molybdate as well as complex I catalyze the reduction of N_2H_4 to NH_3. Similar experiments with complex II revealed that N_2H_4 is also reduced efficiently in the molybdocyanide system; ATP has a significant stimulatory effect. Conversions of 80-90% were reached after 10 min of reaction at 25 °C.

Effects of Ferredoxin Model Compounds. To increase the electron transfer efficiency in the present system we investigated the effect of added salts of the anion $[Fe_4S_4(SR)_4]^{2-}$ (R = n-C_3H_7) on the rate of N_2 reduction. The results in Table V show that the ferredoxin model compound[8] causes an enhancement of the yield. Similar experiments in which iron was added in the form of $FeSO_4$ revealed no significant stimulatory effects.

Effect of Inhibitors. The reduction of N_2 with complex II, $NaBH_4$, and ATP is inhibited by CO, CN^-, C_2H_2, and O_2 (Table VI) all of which also inhibit reduction of coordinated CN^- in the absence of molecular nitrogen as the substrate.

Effects of Other Nucleoside Phosphates and Acid Anions. The reduction of coordinated cyanide in complex II is stimulated by ATP, but less so or not at all by ADP, AMP, SO_4H^-, ClO_4^-, Cl^-, or PO_4H^{2-}. It appeared to be of interest to investigate the effect of the other nucleoside phosphates and acid anions on the reduction of N_2 as well. Table VII indicates that ATP stimulates nitrogen reduction most strongly, ADP and AMP at best marginally, hydrolyzed ATP and the acid anions not at all. It is noteworthy that N_2 inhibits the reduction of coordinated cyanide to hydrocarbons only if ATP is present. In accord with recent[9] work on the ATP effect in molybdothiol systems we conclude that ATP is apparently most effective in converting the molybdenum catalysts into the active reduced forms.

Reduction of CN^- and of N_2 in the MoO_4^{2-}-CN^- System. The reduction of CN^- and of N_2 was investigated in the system MoO_4^{2-}-CN^- to obtain some information on the composition of the catalytically active species generated from complex II under the reaction conditions. The results given in Figure 5 indicate that CN^- reduction occurs optimally at the MoO_4^{2-}:CN^- ratios of between 1:1 and 1:2 and that the reduction of CN^- is inhibited by N_2. Maximum N_2 fixation occurs at the MoO_4^{2-}:CN^- ratio of 1:1. The inhibition of CH_4 production by N_2 reaches a maximum of 28% at the MoO_4^{2-}:CN^- ratio of 1:2; at the same MoO_4^{2-}:CN^- ratio, C_2 hydrocarbon production is inhibited by 59%. The NH_3 production from N_2 reaches a maximum at the MoO_4^{2-}:CN^- ratio of 1:1; some residual N_2H_4 is also detectable, the largest amounts relative to NH_3 were seen at the MoO_4^{2-}:CN^- ratio of 1:2.

Discussion

The present work provides a new example for the reduction of N_2 in homogeneous reaction systems containing catalytically active molybdenum species. Whereas oxomolybdate ions do

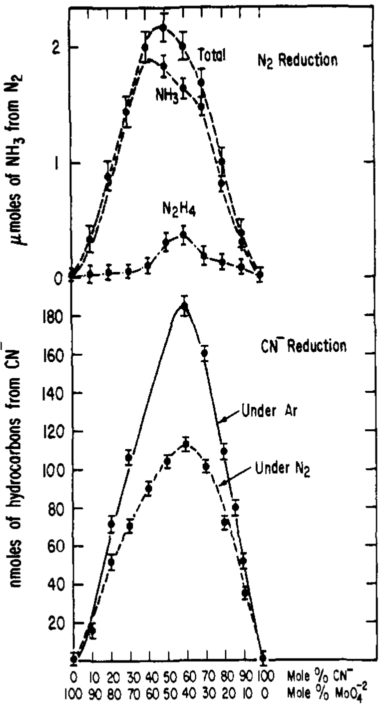
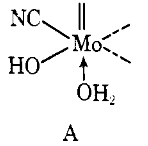


Figure 5. Production of hydrocarbons from CN^- and of NH_3 and N_2H_4 from N_2 in the system MoO_4^{2-}-CN^-. Concentration of MoO_4^{2-} and CN^- at the 1:1 ratio is 0.0054 M; initial concentration of ATP, 0.04 M, of BH_4^-, 0.08 M. Total reaction volume was 4.0 ml. Yields were measured after 1 h of reaction at 20 °C; reactions were stopped by quenching with 1 ml of 6 N HCl.

not catalyze the reaction of N_2-ase substrates efficiently, the coordination of a ligand such as a thiol changes the oxidation-reduction potential of molybdenum sufficiently to produce active systems duplicating virtually all of the reactions of the enzyme. We have previously shown that a number of other ligands, as well as thiols, have a similar effect, and our recent work establishes that this also applies to cyanide. The molybdocyanide systems may be used to study mechanistic details of the catalytic action of molybdenum species in the reduction of substrates of N_2-ase. Complex II may be regarded as a model of a substrate-catalyst complex with respect to the reduction of CN^-, but is not the actual catalyst in the reduction of other substrates. Catalytically active molybdocyanide species are generated from complex II under reducing conditions. It appears that at least two cyanide ions must be removed to produce catalytically active species. The study of CN^- and N_2 reduction in the system MoO_4^{2-}-CN confirms this conclusion and shows that active reduced forms of the catalyst contain one or two cyanide ions per molybdenum. Structures A and B may be assumed for these species in view of the known structure of complex II; they may be considered as analogues of "Mo^{red}" in the molybdothiol systems. With molybdocyanides, N_2 is reduced to NH_3 via N_2H_2 and N_2H_4 just as in the molybdothiol systems; the reaction is significantly ATP stimulated; under otherwise identical conditions, little if any stimulation occurs with ADP, AMP. Evidently, ATP is particularly active and required for the removal of kinetically inert OH^- group(s) from the molybdenum center. We are again led to assume that two cis positions must be available for the catalyst to interact with N_2. The formation of N_2H_2 and its subsequent reactions are schematically represented in eq 2, where Mo^{red} and Mo^{ox} denote the active reduced and oxidized forms of the catalysts A or B; coordinated CN^-, OH^-, or possible complexes of the catalyst with N_2H_2 are not shown. The reduction of N_2 to NH_3 with molybdocyanide catalysts is accelerated by ferredoxin model compounds; the latter promote the transfer of electrons from reductant (e.g., BH_4^-) to Mo^{ox}. Approximate turnover numbers for N_2 reduction may be calculated if the concentration of catalytically active molybdenum species generated from complex II is estimated from the observed yields of CH_4, C_2H_6, and C_2H_4. The turnover numbers calculated in this fashion are 1-3 mol of N_2 reduced to NH_3 per mole of active molybdenum per minute, corresponding to 2-6% of the activity[10,11] of N_2-ase. Under conditions of highest electron transfer efficiency (i.e., after short reaction times), the N_2 turnover is even higher, reaching ca. 10-20% of the activity of the enzyme. (Examples for the turnover number calculations are given in the Experimental Section.) However, the significance of the turnover numbers is somewhat limited due to the relatively short time of optimal functionality. Our studies also show that hydrazine, the disproportionation product of diimide, accumulates in the reaction solutions under certain conditions of reaction and that some of the hydrazine is reduced to ammonia during the acid quenching process. The accumulation of hydrazine is in part due to the fact that the pH of the reaction solutions becomes more alkaline as the reaction proceeds (i.e., BH_4^- is being consumed). Conceivably, pH changes during the reaction may be avoided if an electrochemical method of generation of Mo^{red} is adopted.



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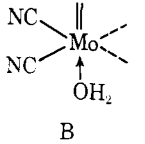

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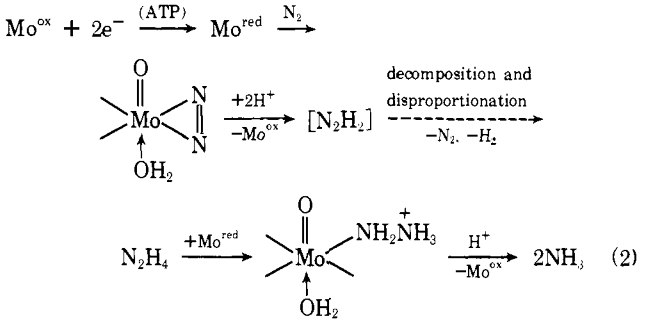

Experimental Section

Reagents and Chemicals. All common reagents and chemicals obtained from commercial sources were of analytical or reagent grade purity and were used without further purification. The salt $K_2[Mo(O)(H_2O)(CN)_4]$ was prepared according to the method of Van de Poel and Neumann.[12] The disodium salts of ATP and ADP and free AMP were purchased from Calbiochem; $NaBH_4$ in the form of 0.25-g pellets was obtained from Ventron.

Standard Experimental Technique for N_2 Reduction Experiments. The reduction of N_2 was performed by a standard technique similar to that outlined in ref 1. Reaction flasks of 38 ml total capacity, manufactured by Pierce Chemical Co., Rockford, Ill., were sealed with silicone rubber seals and flushed with pure normal N_2 (99.991% purity) for 20 min. After the removal of an equivalent amount of gas from the reaction flask by means of a syringe, 20 ml of gaseous N_2 (at 1 atm), containing 50.5% $^{30}N_2$ and 50.0% $^{28}N_2$ (obtained from International Chemical and Nuclear Corp., Irvine, Calif.), was injected, followed by 1.5 ml of a 0.014 M soluion of complex II in 0.2 M, pH 7.5 borate buffer. Subsequently, 0.7 ml of 1 M NaOH was added. The ATP (0.5 ml of a freshly prepared, unbuffered 0.58 M aqueous solution) was injected next. The pH of the resulting solution is 7.5. Occasionally, pH-adjusted ATP solutions were also employed, i.e., by adding 1 M NaOH to the ATP solution prior to its injection into the reaction flask. To minimize ATP hydrolysis during the pH adjustment, cooling with ice is recommended. It is also critical that this operation is performed as rapidly as possible; if the ATP hydrolyzes at this stage, N_2 fixation is not observed. Immediately after the ATP addition, 0.5 ml of a freshly prepared 0.66 F $NaBH_4$ solution was injected to initiate the reaction. The gas pressure was maintained at 1 atm by means of a 50-ml syringe (with initially fully depressed piston) attached to a vent needle. After reaction at the given temperatures and reaction times, the reactions were stopped either by freezing with liquid N_2 or by injection of acid (e.g., 1 ml of 6 N HCl). At this point, 0.5-ml gas samples were withdrawn for hydrocarbon analysis by GPC. Also 1-ml aliquots of the reaction solutions were transferred into one compartment of a Rittenberg flask, degassed, and oxidized with freshly prepared alkaline sodium hypobromite as outlined in ref 13. The N_2 formed was collected by means of a Toepler pump and analyzed for $^{28}N_2$, $^{29}N_2$, and $^{30}N_2$.[14] The yield of $^{29}N_2$ was corrected for natural abundance of $^{29}N_2$ from the observed $^{28}N_2$ peak, assuming the natural abundance of $^{29}N_2$ to be 0.74%. Absolute yields of fixed nitrogen were obtained by comparison of the observed peak heights with those of $^{30}N_2$ as released upon hypobromite oxidation of a standard solution of 95.8% $^{15}NH_4Cl$, and were recalculated to correspond to 100% $^{30}N_2$ as the substrate.

The results of the nitrogen fixation experiments with isotopically enriched N_2 were confirmed by colorimetric measurements of the yields of NH_3 and N_2H_4 as outlined in ref 1. Additional details of experiments under different conditions are given in the legends to figures and tables. The reproducibility of the experiments is good, provided that care is taken to avoid contamination of the gas phase by oxygen and that pure $NaBH_4$ and ATP are used. Only fresh samples of the latter are active and should be dissolved immediately before the experiment. For the colorimetric determination of NH_3, the total yield of NH_3 observed must be corrected for background NH_3 by performing blanks under argon.[15]

Studies on the System MoO_4^{2-}–CN^-. For the studies of N_2 fixation in the system MoO_4^{2-}–CN^-, reaction flasks were filled with $^{30}N_2$-enriched nitrogen and subsequently mixtures of MoO_4^{2-} and CN^- in varying molar ratios were prepared by injecting the appropriate volumes of 0.046 M solutions of Na_2MoO_4 and KCN, respectively, both of which were dissolved in 0.2 M pH 7.5 borate buffer. To the individual reaction flasks, 0.5 ml of 0.5 M ATP and 0.5 ml of 0.66 M $NaBH_4$ solution were injected to initiate the reaction. Workup and analysis were the same as described above.

Reduction of N_2H_4. The reduction of N_2H_4 was investigated in the system MoO_4^{2-}–CN^- as described above except that the reaction flasks were filled with pure argon and 1 ml of a solution containing 6.2 μmol of N_2H_4 was injected prior to the addition of ATP and $NaBH_4$. The course of the reactions was followed by determining the concentrations of N_2H_4 and NH_3 colorimetrically (see ref 1, 5) in aliquots withdrawn at certain time points.

Calculation of Approximate Turnover Numbers of N_2 Reduction. A solution containing 42 μmol of complex II reduces 3.3 μequiv of N_2 to NH_3 and N_2H_4 during the first 20 min of reaction at 20 °C. Simultaneously, 0.15 μmol of CN^- are reduced to hydrocarbons (CH_4, C_2H_4, and C_2H_6). Assuming that two CN^- must be removed from complex II to afford one molecule of catalytically active species for N_2 reduction, this means that 0.075 μmol of that species reduced 1.65 μmol of N_2 in 20 min. Accordingly, 1.1 mol of N_2 are reduced per mole of active molybdenum catalyst per minute, corresponding to 2.2% of the turnover number of N_2-ase. During the first 6 min of reaction, 6 μequiv of reduced N_2 in the form of NH_3 and N_2H_4 are found on workup of the reaction solution (see Figure 3). At this time-point, 0.1 μmol of CN^- is reduced to hydrocarbons, corresponding to 0.05 μmol of active molybdenum catalyst. The turnover number is thus calculated to 10 (moles of N_2 reduced per mole of active catalyst per minute), which is 20% of the turnover number of N_2-ase.

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(14) The mass spectrographic assays were performed using an LKB 9000 Model spectrograph.
(15) **Note Added in Proof:** Distillation of alkaline reaction solutions which were previously treated with excess $HgCl_2$ to trap CN^- has since been found to eliminate CN^- interference in the colorimetric NH_3 determinations more effectively than the procedure outlined in ref 1. The absolute yields observed by both methods are equal within experimental error.