The Chemical Evolution of a Nitrogenase Model. V. The Reduction of Nitriles[1]

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Abstract: Nitrogenase model systems, composed of molybdate and thiol ligands such as, *e.g.*, cysteine, catalyze the reduction of aliphatic nitriles to alkanes and NH_3 in the presence of $NaBH_4$. The reaction is significantly stimulated by substrate amounts of ATP, is faster in D_2O than in H_2O, and is inhibited up to 90 and 41%, respectively, by CO and N_2 at 1 atm of partial pressure. Experiments with $^{15}N_2$ indicate that molecular nitrogen is reduced under these conditions, while CO apparently inhibits by forming a complex with the catalyst. The catalytically active species in the model systems are mononuclear molybdenum–thiol complexes. In the active reduced form molybdenum is probably in the 4+ oxidation state. The ATP stimulates the reduction of nitriles and of other substrates by accelerating the conversion of oxidized (*i.e.*, Mo(V) and Mo(VI)) forms of the catalyst complexes to the active reduced form, by providing a better leaving group for the relatively inert molybdenum-bound OH groups. The initial interaction of the substrate with the catalyst is assumed to involve side-on bonding of the CN group, giving rise to the formation of an organomolybdenum species, whose subsequent reactions occur without Mo–C bond cleavage, until a terminal alkylmolybdenum complex is formed, whose hydrolysis yields the saturated hydrocarbon product plus the oxidized form of the catalyst. The reduction of unsaturated nitriles affords mixtures of alkanes and olefins in analogy to the reactions catalyzed by *Azotobacter vinelandii* nitrogenase. The olefinic organomolybdenum intermediates in this case undergo double bond and cis–trans isomerization prior to the hydrolysis of the molybdenum–carbon bond. The types of reaction of unsaturated nitriles are exemplified in detail for *cis*- and *trans*-crotononitrile as the substrate, also with catalyst systems containing 2-aminoethanethiol, glutathione, and bovine serum albumin as the thiol component. Since all previously observed reactions of nitriles with nitrogenase are essentially duplicated by the model systems, nitrile binding and reduction by nitrogenase must occur at the molybdenum active site of the Fe–Mo–protein of mol wt 270,000. Iron, which cannot replace molybdenum in the model system, exerts a modest cocatalyst effect. Its primary function in nitrogenase appears to be the activation of electron transport to the molybdenum active site.

Azotobacter vinelandii nitrogenase (N_2-ase) reduces saturated, unbranched nitriles up to, and including, *n*-butyronitrile, to alkanes plus ammonia (eq 1).[2-5]

$$\text{R-CN} + 6\text{e} \xrightarrow{6\text{H}^+,\ \text{ATP},\ \text{Mg}^{2+},\ \text{nitrogenase}} \text{R-CH}_3 + \text{NH}_3 \quad (1)$$

The nitrile reduction is ATP dependent just as the reduction of nitrogen and of the other substrates of N_2-ase, but is quite slow, only about 0.5% of the rate of reduction of nitrogen. Acrylonitrile is a better substrate and is reduced to a 6:1 mixture of propylene and propane at about 50% of the rate of nitrogen.[3] Although branched aliphatic nitriles are not reduced, methacrylonitrile is slowly converted into a mixture of isobutylene and isobutane.

Since nitriles are isoelectronic with molecular nitrogen, elucidation of their mechanism of reduction in plausible model systems is prerequisite to the understanding of biological nitrogen fixation.

The results of the enzymatic studies were interpreted to suggest that the reduction of nitriles takes place at the same active site or sites involved in the binding and reduction of nitrogen. It is still unknown, however, if the active site or sites consist of molybdenum, iron, or both metals attached to the apoprotein. It has been suggested, however, that nitrogen is first bound by iron, and reduced to ammonia *via* an intermediate complex containing the unit Fe–N_2–Mo.[3,4] In papers II and IV of this series, model studies were reported which suggest that reactions of nitrogenase with acetylenes and isonitriles are characteristic of a molybdenum binding and reduction site, and that the iron present in the enzyme probably acts as an electron transfer catalyst.[6,7] We have since extended our studies to the reduction of aliphatic and olefinic nitriles and report the results of this work in the present paper.

Results

Model Systems Employed. The N_2-ase model systems employed were identical with those used previously,[6,7] consisting either of molar 1:1 mixtures of L(+)-cysteine (Cys) and MoO_4^{2-}, or the binuclear Mo(V) complex $Na_2Mo_2O_4(Cys)_2 \cdot 5H_2O$ (complex I). The catalytic activity of freshly prepared MoO_4^{2-}–Cys mixtures is greater than that of equivalent solutions of complex I, which as such is not active. Complex I is known to dissociate in solution into monomeric species, however, which in the presence of $NaBH_4$ are converted to the catalytically active reduced form. Conversely, MoO_4^{2-}–Cys solutions on standing in the presence of a

(1) Communicated, in part, at the Annual Meeting of the Chemical Society, April 10–14, 1972, Manchester, England.

(2) R. W. F. Hardy and E. K. Jackson, *Fed. Proc., Fed. Amer. Soc. Exp. Biol.*, 26, 725 (1967).

(3) (a) R. W. F. Hardy, R. C. Burns, and G. W. Parshall, *Advan. Chem.*, 100, 219 (1971); (b) R. W. F. Hardy, R. C. Burns, and G. W. Parshall, "Bioinorganic Chemistry," G. Eichhorn, Ed., Elsevier, Amsterdam, The Netherlands, 1972; (c) R. W. F. Hardy and E. Knight, Jr., *Progr. Phytochem.*, 1, 407 (1968).

(4) W. H. Fuchsman and R. W. F. Hardy, *Bioinorg. Chem.*, 1, 197 (1971).

(5) Direct proof of the origin of ammonia in eq 1 is difficult due to the simultaneous hydrolysis of the nitrile substrates under the reaction conditions and the slow rate of reduction of nitriles by N_2-ase. Since the nitrile nitrogen atom is already in the 3− valence state the nitrile carbon atom actually accepts the six electrons on reduction.

(6) G. N. Schrauzer and P. A. Doemeny, *J. Amer. Chem. Soc.*, 93, 1608 (1971).

(7) G. N. Schrauzer, P. A. Doemeny, G. W. Kiefer, and R. H. Frazier, *ibid.*, 94, 3604 (1972).

reducing agent lose catalytic activity mainly due to the formation of inactive oxo-bridged Mo(V) dimers identical with, or similar to, complex I. Since the activity of the model systems is sensitively influenced by minor variations of the experimental conditions, rate data quoted should therefore be viewed as typical rather than absolute. The catalytic activity of the model systems also depends to some extent on the nature of the thiol component; although Cys does not convey the highest catalytic activity of all ligands, it was chosen to obtain results comparable with ref 6 and 7. For similar reasons $NaBH_4$ was selected as the reducing agent. Although $Na_2S_2O_4$ may also be used in our system, it is too inefficient for the reduction of nitriles. Most experiments were conducted in pH 9.6–10 buffered solutions, close to the established optimal pH conditions. The highest catalytic activity for nitrile reduction was obtained using systems containing MoO_4^{2-}, Cys, catalytic amounts of Fe^{2+} (supplied as $FeSO_4 \cdot 7H_2O$),[8] excess $NaBH_4$, and substrate amounts of freshly dissolved ATP. The catalytic activity of this complete system and the effects of various omissions are summarized in Table I. The omission of the Fe^{2+} cocatalyst causes only a small loss of activity. Hence the most active "minimum component system" for CH_3CN reduction consists of MoO_4^{2-}, Cys, ATP, and $NaBH_4$ (system no. 2 in Table I); unless specifically indicated most experiments were conducted with this system, or by replacing MoO_4^{2-} and Cys with equivalent amounts of complex I, in the absence of added iron cocatalyst. The system produces ethane from acetonitrile at a constant rate during the first 30 min of reaction (Figure 1). All rates quoted in this paper were obtained from measurements during this period.

Table I. Relative Rates of Ethane Production from Acetonitrile[a]

No.	System	Initial rates of C_2H_6 formation nmol/min	Rel
1	Complete	0.532	100
2	1, $-Fe^{2+}$	0.475	84
3	2, −ATP	0.087	15.4
4	3, +boiled ATP	0.109	21.3
5	2, $-MoO_4^{2-}$	0.016	3.2
6	2, −Cys	0.025	5.0
7	2, $-BH_4^-$	0	0
8	2, $-CH_3CN$	0	0
9	−, BH_4^- + ATP	0.006	1.2
10	−, BH_4^-	0.003	0.6

[a] The complete system contained the following components in a total volume of 4.7 ml: MoO_4^{2-}, 0.0037 mmol; Cys, 0.0037 mmol; Fe^{2+}, 0.30 μmol; $NaBH_4$ (initial), 0.67 mmol; ATP (initial), 0.6 mmol; CH_3CN (initial), 1.90 mmol.

ATP is required in substrate rather than catalytic amounts and is consumed during the reaction. Boiled ATP solution is ineffective. Among other phosphates studied GTP proved to be about as effective as ATP. ADP and AMP are progressively less active, creatine phosphate is inactive, and $Na_5P_3O_{10}$ is inhibitory (Table II). Incidentally, methane is formed in trace amounts from acetonitrile with noncrystallized commercial $NaBH_4$. The amount of CH_4 decreases upon recrystallization of $NaBH_4$ from diglyme and also if EDTA is added to the reaction solution. Presumably a cleavage of the C–CN bond is catalyzed by traces of heavy metal contaminants in the $NaBH_4$; reactions of this type have recently been observed in nonaqueous systems using iron complexes as catalysts in the presence of, *e.g.*, sodium naphthalenide.[9] All results of experiments quoted in this paper are corrected for this background hydrocarbon formation, which actually diminishes further upon adding the molybdothiol catalyst.

(8) The cocatalyst effect of iron is evident only within the first 5–10 min after initiation of the reaction and only at concentrations of 5% of the total molybdenum present. Higher levels of iron are inhibitory; for similar observations in the reduction of acetylenes and isonitriles, see ref 6 and 7.

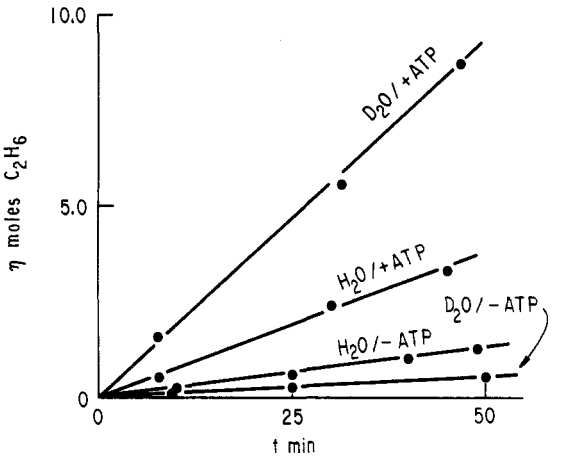


Figure 1. Time dependence of C_2H_6 formation from CH_3CN with complex I–$NaBH_4$ in H_2O and D_2O, in the presence and absence of ATP, respectively. The reaction vessels contained the substrates in a total volume of 3.70 ml of 0.2 *M* pH 9.6 borate buffer and the following initial amounts: complex I, 7.5×10^{-3} mmol; CH_3CN, 3.82 mmol; $NaBH_4$, 0.67 mmol; ATP, 0.60 mmol.

Table II. Effects of Various Phosphates on the Catalytic Reduction of CH_3CN[a]

Addition	C_2H_6, nmol	% stimulation
None	0.60	
ATP	6.0	1000
GTP	6.0	1000
ADP	3.36	560
AMP	2.86	477
Adenine	0.60	0
Creatine phosphate	0.61	0
$Na_5P_3O_{10}$	0.54	0

[a] System contained 0.0075 mmol, complex I; 0.67 mmol, $NaBH_4$ (initially) in a total volume of 3.7 ml, to which 1.0 ml of freshly prepared solution (0.1 *M*) of the phosphorylating agents was added.

Characteristics of the Catalytic Nitrile Reduction. The ethane production from acetonitrile with complex I–$NaBH_4$ increases approximately linearly with [complex I]$^{1/2}$ up to the concentration of 1.0×10^{-4} *M* without ATP, and 4.0×10^{-4} *M* with ATP, indicating that the catalytically active species is monomeric. The rate of ethane formation decreases at higher concentrations of complex I (Figure 2). In the presence of substrate amounts of ATP the rate of ethane production is accelerated by a factor of 2.6 in D_2O relative to H_2O as the solvent. In the absence of ATP, the rate of ethane production in D_2O is approximately one-third of that in H_2O (Figure 1). The rate of acetonitrile reduction increases linearly with [CH_3CN] up to 0.6 *M;* substrate

(9) E. E. van Tamelen, H. Rudler, and C. Bjorklund, *J. Amer. Chem. Soc.*, 93, 7113 (1971).

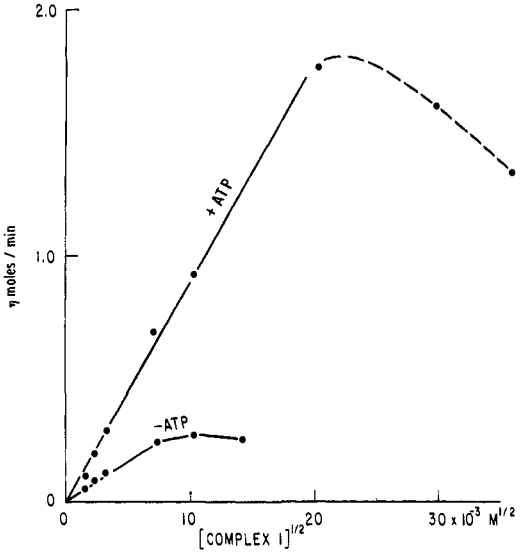


Figure 2. Dependence of the initial rate of C_2H_6 production from CH_3CN on the concentration of complex I, in the presence and absence of ATP. The total solution volume was 3.65 ml, containing 1.91 mmol of CH_3CN, 0.67 mmol of $NaBH_4$, 0.06 mmol of ATP (initially), in 0.2 *M* pH 9.6 borate buffer.

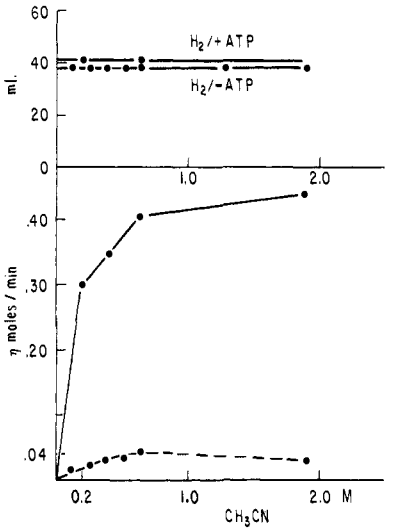


Figure 3. Dependence of the rate of C_2H_6 production from CH_3CN, and of the amount of H_2 produced, on $[CH_3CN]$. Initial amounts of reagents present: complex I, 0.015 mmol; $NaBH_4$, 0.67 mmol; ATP, 0.06 mmol. Total volume 3.95 ml; solvent, 0.2 *M* pH 9.6 borate buffer.

inhibition of the reaction occurs at higher concentrations (Figure 3). The rate of ethane production from acetonitrile is an approximately linear function of the initial ATP concentration up to 0.14 *M*, beyond which it becomes inhibitory (Figure 4). The apparent E_a of CH_3CN reduction in H_2O is 16.2 kcal in the absence of ATP, and 13.2 kcal in the presence of substrate amounts of ATP, in the temperature range between 15 and 40°. From Lineweaver–Burk plots the K_m for the reduction of CH_3CN was estimated to be around 1000 m*M*. The reduction of CH_3CN is accompanied by H_2 evolution (from the hydrolysis of BH_4^-), which, in part, is catalyzed by the molybdate–Cys catalyst. The H_2 evolution is significantly inhibited with acetylene[6] as the substrate, but not noticeably by CH_3CN (Figure 3).

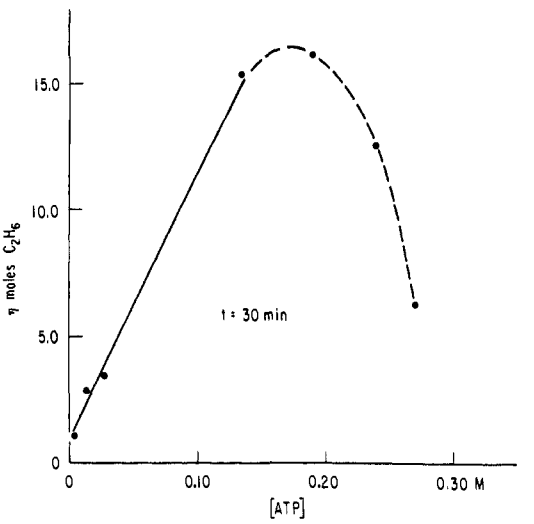


Figure 4. Dependence of C_2H_6 production from CH_3CN on the ATP concentration. Reaction vessels contained a total volume of 3.70 ml of 0.2 *M* pH 9.6 borate buffer with the reactants in the following initial concentrations: complex I, 0.0068 mmol; CH_3CN, 3.82 mmol; $NaBH_4$, 0.67 mmol.

The reduction of CH_3CN by complex I–$NaBH_4$ is reproducibly inhibited by CO; at optimal concentrations of substrate, catalyst, and 1 atm of partial pressure, the inhibition is 90% in the absence of ATP. In the presence of ATP the inhibition by CO decreases to 66%. Nitrogen at 1 atm of partial pressure inhibits 11% relative to 1 atm of argon in the absence of ATP; in the presence of ATP N_2 inhibition *increases* to 41%. Experiments with $^{15}N_2$ under these conditions indicate that molecular nitrogen is reduced to ammonia, as determined by mass spectrographic analysis of the hypobromite oxidized reaction mixture. Thus, 0.22 μmol of NH_3 arising from $^{15}N_2$ was formed in 0.1 *N* solutions of CH_3CN with complex I–$NaBH_4$–ATP after 48 hr of reaction at room temperature. In the absence of CH_3CN, this system under otherwise identical conditions produces 0.58 μmol of NH_3 through fixation of molecular N_2.

Activity of Metals Other Than Molybdenum. The reduction of CH_3CN to C_2H_6 was not observed when molybdenum in the MoO_4^{2-}–Cys system was replaced by ions of the metals Fe, Cu, V, Zn, Cd, Cr, Ti, Zr, Hf, Mn, Pd, Ru, and Rh (reductant $NaBH_4$), with and without substrate amounts of ATP. Active systems resulted with WO_4^{2-}, Co^{2+}, and Ni^{2+}. In contrast to the corresponding systems with molybdenum, the reduction of CH_3CN was not stimulated, and in some cases was inhibited by ATP. With these metals little inhibition by N_2 was observed, which diminished further in the presence of ATP. Inhibition by CO was more significant, ranging between 39 and 92% (Table III).

Table III. Relative Rates of C_2H_6 Production from CH_3CN[a]

Metal	Rel rate of C_2H_6 production		% inhibition by N_2		% inhibition by CO	
	−ATP	+ATP	−ATP	+ATP	−ATP	+ATP
MoO_4^{2-}	1.0	8.8	11	41	90	66
WO_4^{2-}	0.93	0.96	9.7	9	48	39
Co^{2+}	5.77	1.02	13	0	77	92
Ni^{2+}	0.66	0.52	12.3	9	79	67

[a] Reaction conditions as given in legend to Figure 1.

Table IV. Products and Initial and Relative Rates of Reduction of Various Nitriles by Complex I–$NaBH_4$ in the Absence and Presence of ATP

Substrate	Products	k_{init}, nmol/min −ATP	k_{init}, nmol/min +ATP	k_{rel} −ATP	k_{rel} +ATP
CH_3CN	C_2H_6	0.080	0.62	1.00	7.75
CH_3CH_2CN	C_3H_8	0.034	0.34	0.42	4.25
$CH_3CH_2CH_2CN$	C_4H_{10}	0.070	0.284	0.88	11.0
$(CH_3)_3CHCN$	i-C_4H_{10}	0.092	0.419	1.15	14.4
$(CH_3)_3CCN$	C_5H_{12}	~0.001	~0.01	~0.125	~1.50
CH_2=$CHCN$	C_3H_8, C_3H_6	0.120	0.89	1.50	18.7
cis-CH_3CH=$CHCN$	n-C_4H_{10}, 1-C_4H_8; 2-C_4H_8	0.15	1.6	1.87	20.0
trans-CH_3CH=$CHCN$	n-C_4H_{10}, 1-C_4H_8; 2-C_4H_8	0.15	1.6	1.87	20.0
CH_2=$CH(CH_3)CN$	i-C_4H_{10}, i-C_4H_8	0.14	1.3	1.75	16.2

Table V. Rates and Product Ratios of the Complex I Catalyzed Reduction of Acrylonitrile and of *cis*- and *trans*-Crotononitrile

Substrate	Conditions	Product ratios				Initial rates, nmol/min
CH_2=$CHCN$		C_3H_8	C_3H_6			
	H_2O	1.00	1.50			0.03
	D_2O	1.00	1.24			0.02
	H_2O, ATP	1.00	1.18			1.10
	H_2O, ATP, Fe^{2+}	1.00	1.00			1.00
	D_2O, ATP	1.00	1.50			3.20
	D_2O, ATP, Fe^{2+}	1.00	1.43			3.34
cis-CH_3CH=$CHCN$				Trans	Cis	
		C_4H_{10}	1-C_4H_8	2-C_4H_8	2-C_4H_8	
	H_2O	1.00	3.66	Trace	Trace	0.04
	D_2O	1.00	4.00	Trace	Trace	0.008
	H_2O, ATP	1.00	0.50	Trace	Trace	0.51
	H_2O, ATP, Fe^{2+}	1.00	0.91	0.13	0.05	0.57
	D_2O, ATP	1.00	0.59	0.04	0.09	1.23
	D_2O, ATP, Fe^{2+}	1.00	0.78	0.12	0.06	1.55
trans-CH_3CH=$CHCN$						
	H_2O	1.00	1.35	0.18	Trace	0.07
	D_2O	1.00	2.75	0.11	Trace	0.02
	H_2O, ATP	1.00	0.97	0.23	0.07	1.60
	H_2O, ATP, Fe^{2+}	1.00	0.97	0.24	0.07	1.70
	D_2O, ATP	1.00	0.80	0.23	0.08	2.14
	D_2O, ATP, Fe^{2+}	1.00	0.80	0.23	0.07	2.94

It appears that the systems containing nickel are not genuinely homogeneous, possibly containing finely divided or colloidal nickel.

Substrate Variation. Products and initial rates of reduction for various substrates are summarized in Table IV. The reduction of acrylonitrile and of *cis*- and *trans*-crotononitrile was investigated in H_2O and D_2O, in the presence or absence of substrate or catalytic amounts of ATP and iron, with complex I as catalyst, or with catalyst systems composed of 1:1 mixtures of MoO_4^{2-}–Cys, MoO_4^{2-}–glutathione (GSH), MoO_4^{-2}–2-aminoethanethiol[10] and MoO_4^{2-}–bovine serum albumin (BSA). Results are summarized in Tables V–VII. It is important to point out that the rates and product ratios differ depending on whether complex I or MoO_4^{2-}–Cys catalysts are employed. However, the product ratios remain essentially invariant during the first 60 min of reaction. In the experiments with substrate amounts of ATP the reaction was found to proceed rapidly during the first 10 min and after this time essentially terminated. Rate data quoted in the tables referring to runs in the presence of ATP were measured during the observed period of activity; those in the absence of ATP were recorded after 60 min of reaction to increase the accuracy of measurement.

(10) Note Added in Proof. Results obtained with 2-aminoethanethiol (AET) are included to show that this ligand does not offer substantial advantages over cysteine. R. E. E. Hill and R. L. Richards (*Nature (London)*, 233, 114 (1971)), in contrast, reported data which suggest that AET in conjunction with MoO_4^{2-} and Fe^{2+} as the cocatalyst is far superior for nitrogen fixation than our model systems containing cysteine. We wish to point out that Hill and Richards' experiments with $^{15}N_2$ and the AET–MoO_4^{2-} catalyst system are irreproducible (personal communication by Dr. Richards). We also found that AET–MoO_4^{2-} systems fix less nitrogen than those containing Cys, and that the presence of Fe^{2+} is not absolutely essential. The seemingly high yields of nitrogen fixed by AET–MoO_4^{2-} systems are caused by the decomposition of AET under the reaction conditions, giving rise to NH_3. After appropriate correction for natural abundance of ^{15}N, the system turns out to be considerably less active than MoO_4^{2-}–Cys catalysts employed by us. Details of this work will be described in a forthcoming paper.

Discussion

The reduction of a nitrile to a methyl group is a six-electron process which most likely occurs *via* organomolybdenum intermediates. The nitrile may be assumed to interact initially with the active reduced Mo–Cys complex of probable structure **1**, either by forming an "end-on" complex **2** or by attaching itself "side-on" as in **3**. A plausible pathway for ethane formation from acetonitrile may be written by assuming **3** to be the initial catalyst–substrate adduct (Mo_{red} designates **1**, Mo_{ox} the corresponding Mo(VI) form of the catalyst; molybdenum-bound hydroxyl groups are omitted).

Intermediates **5** and **6** in eq 2 are identical with those postulated in the reduction of isonitriles.[7] The formation of organomolybdenum intermediates would be

Table VI. Rates and Product Distribution in the Reduction of *cis*- and *trans*-Crotononitrile with Catalyst Systems Composed of 1:1 Mixtures of MoO_4^{2-} with L(+)-Cysteine (Cys)[a]

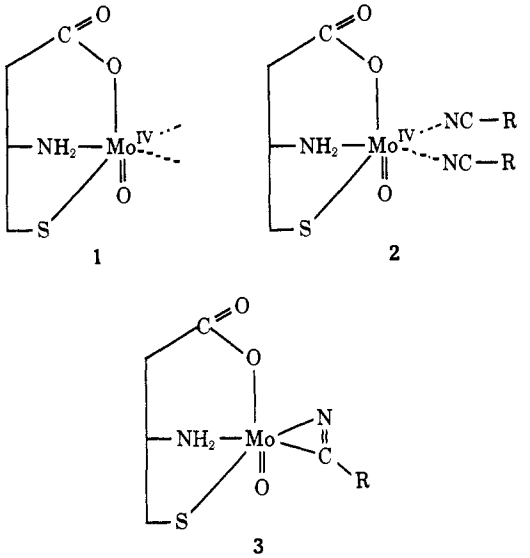
	cis-Crotononitrile[b]				*trans*-Crotononitrile[b]			
Solvent	H_2O	H_2O	D_2O	D_2O	H_2O	H_2O	D_2O	D_2O
ATP	−	+	−	+	−	+	−	+
Initial rate, nmol/min	0.526	2.59	0.062	8.48	0.808	14.5	0.041	19.6
Product distribution								
n-C_4H_{10}	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
1-C_4H_8	0.77	0.99	1.23	0.78	0.82	1.31	1.46	1.11
trans-2-C_4H_8	0.50	0.14	0.93	0.09	0.49	0.25	0.70	0.23
cis-2-C_4H_8	0.09	0.12	0.11	0.13	0.08	0.11	0.09	0.07

[a] In the presence and absence of ATP, and in H_2O and D_2O. Reaction solutions were 0.005 *M* in MoO_4^{2-} and Cys in 3.0 ml of pH 9.6 borate buffer (0.2 *M*); initial amount of substrate, 1 mmol; of ATP, 0.4 mmol. [b] Substrate.

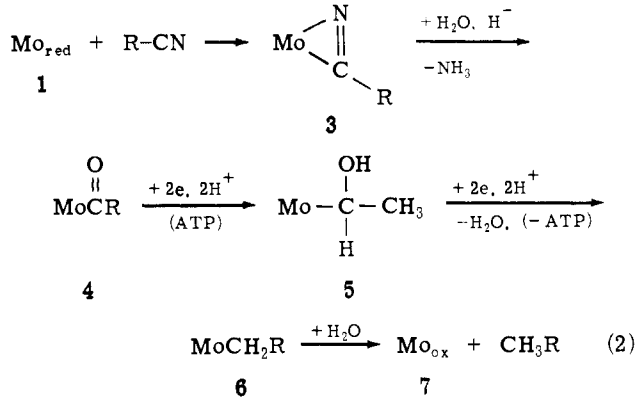
Table VII. Rates and Product Distribution in the Reduction of *cis*- and *trans*-Crotononitrile with Catalyst Composed of 1:1 Mixtures of MoO_4^{2-}[a]

	cis-Crotononitrile[b]				*trans*-Crotononitrile[b]			
Thiol[c]	Cys	GSH	BSA	AET	Cys	GSH	BSA	AET
Initial rate, nmol/min	2.59	14.7	4.84	4.21	14.5	16.7	15.07	7.19
Relative rate	1.00	5.7	1.87	1.63	5.6	6.5	5.82	2.78
Product distribution								
n-C_4H_{10}	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
1-C_4H_8	0.99	0.59	1.31	0.92	1.31	0.81	1.60	1.07
trans-2-C_4H_8	0.14	0.09	0.15	0.14	0.25	0.35	0.44	0.25
cis-2-C_4H_8	0.12	0.19	0.22	0.21	0.11	0.09	0.15	0.08
Cis/trans	0.86	2.25	1.49	1.50	0.44	0.25	0.35	0.31

[a] With Cys, glutathione (GSH), bovine serum albumin (BSA), and 2-aminoethanethiol (AET), in the presence of ATP, in H_2O. Reaction time, 15 min. [b] Substrate. [c] Concentrations of MoO_4^{2-}, GSH, BSA, AET (prior to addition of 0.5 ml of 1.33 *M* $NaBH_4$ and 0.25 ml of 1.2 *M* ATP): 0.005 *M* in 3.0 ml of 0.2 *M* pH 9.6 borate buffer.

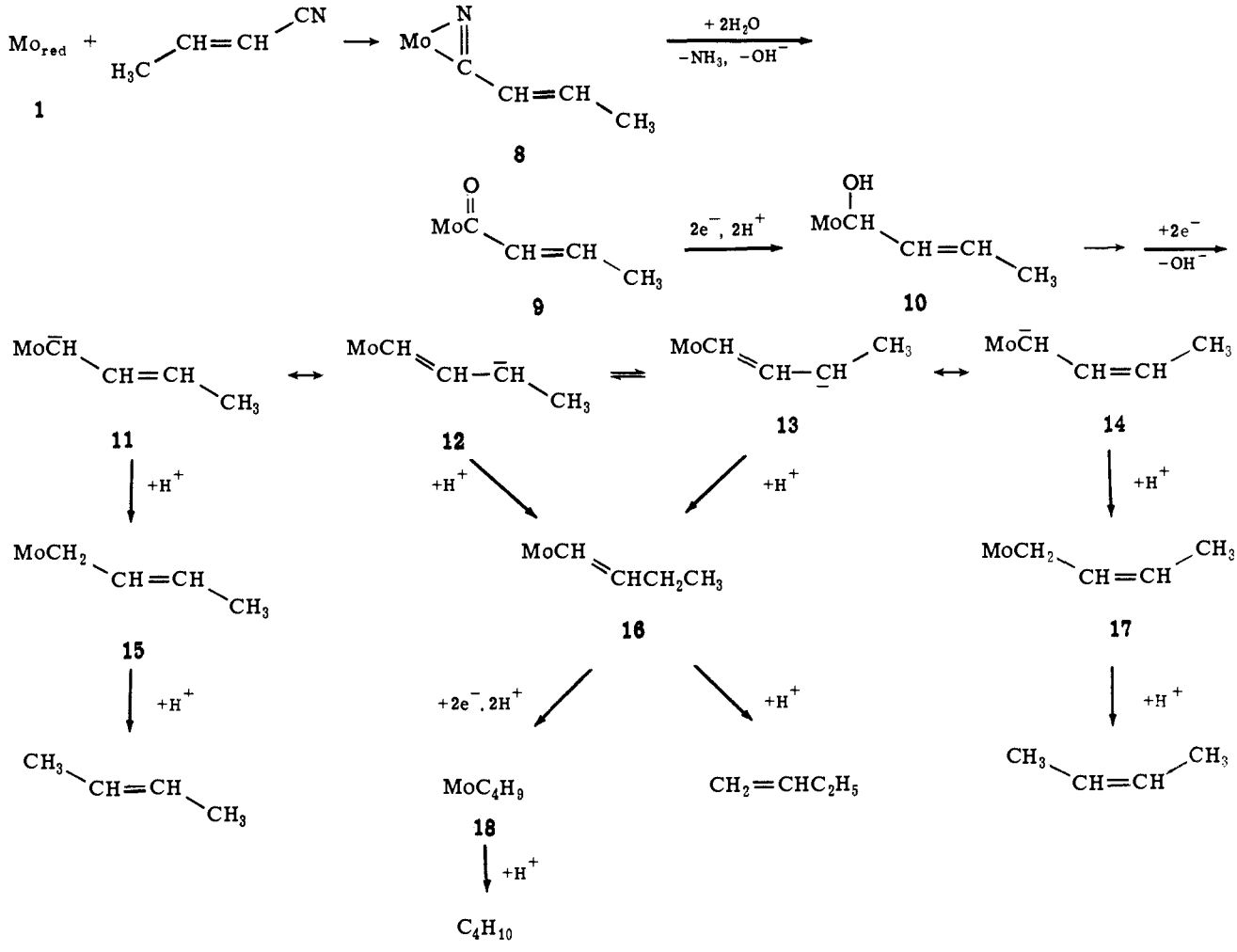


much more difficult to rationalize with **2** as the initial substrate–catalyst complex. Although end-on bonding of the nitriles to one or two coordination sites of the reduced or oxidized form of the catalyst may occur, these need not be necessarily reducible. Conceivably, this type of interaction is responsible for the observed inhibition of nitrile reduction at high substrate levels. The formation of a side-on adduct is expected to be particularly favored if two vacant cis positions at molybdenum are available. The inhibiting effect of carbon monoxide could thus be plausibly attributed to the



blockage of one coordination position by end-on bonded CO. In the presence of ATP the active reduced form of the catalyst is generated in higher stationary concentration, thus favoring the side-on interaction with the substrate. It is of interest in this context that nitrogen is a weak inhibitor of nitrile reduction in the absence of ATP, and that its inhibiting effect increases markedly in the presence of ATP. Carbon monoxide, in contrast, is a stronger inhibitor in the absence of ATP (Table V). The differences in the behavior of N_2 and CO could imply that CO interacts with the catalyst end-on, whereas N_2 binds side-on. The observed very slow rate of reduction of trimethylacetonitrile indicates that the nitrile carbon atom must be exposed to be reducible, as would be expected for a mechanism involving side-on bonding of the C≡N group to the catalyst. On the other hand, *tert*-butyl

Scheme I



isocyanide is readily reduced by our catalyst system,[7] since isonitriles interact with the molybdenum catalyst in the end-on fashion. Complexes of transition metals in which nitriles are presumably bound side-on (*i.e.*, with succinonitrile as the ligand) have been described.[11]

The diminished rate of substrate reduction in D_2O in the absence of ATP is attributed to the deuterium effect on the rates of all intermediate reaction steps involving the solvent. The acceleration of nitrile reduction in D_2O in the presence of ATP is probably due to the more favorable competition of substrate reduction relative to the discharge of the D_3O^+ ion and, in part, the slower rate of hydrolysis of ATP in D_2O. The function of ATP is seen to consist in the acceleration of the reduction of Mo(VI) and Mo(V) forms of the catalyst.[6,7] This is achieved by the phosphorylation of molybdenum-bound OH groups, causing their more facile removal. Conceivably, ATP specifically increases the stationary concentration of the unsolvated active reduced form **1** of the catalyst. If side-on bonding of the nitrile as in **3** is assumed to be the first step of the reaction, this could explain the significant stimulation of nitrile reduction by ATP. ATP may furthermore be expected to accelerate the reduction of all intermediate species containing molybdenum-bound hydroxyl groups. Since the addition of a hydroxyl ion to the molybdenum ion may occur after each transfer of an electron to the bound partially reduced substrate, up to six molecules of ATP may be required for a six-electron reduction. The number of ATP molecules consumed per molecule of substrate reduced need not be constant, however, and may vary with the efficiency of the electron transfer system. Molybdenum appears to be the only metal whose catalytic activity in nitrile reducing systems is significantly stimulated by ATP under nonenzymatic conditions.

In the reduction of α-unsaturated nitriles mixtures of alkanes and olefins are formed. The relative yields of alkanes increase in the presence of ATP as well as in D_2O relative to H_2O, presumably due to the more rapid or efficient reduction of the catalyst under these conditions. Acrylonitrile and crotononitrile are reduced more rapidly than aliphatic nitriles. In the reduction of the olefinic nitriles unsaturated organomolybdenum intermediates are formed which undergo allylic-type double bond and cis–trans isomerizations prior to the terminal Mo–C hydrolysis steps. A plausible reaction scheme is shown in Scheme I. Scheme I contains only σ-bonded intermediates. This does not exclude the possibility that π-allylic organomolybdenum species are involved.

With MoO_4^{2-}–Cys catalysts in the presence of ATP the product distribution of the olefinic species is 1-butene > *trans*-2-butene > *cis*-2-butene, and thus is in the correct order of decreasing thermodynamic stability. The product distribution depends on various reaction parameters, and, in particular, the nature of the thiol

(11) M. F. Farona and N. J. Bremer, *J. Amer. Chem. Soc.*, **88**, 3735 (1966).

Table VIII. Characteristics of Reactions of Nitrogenase and of the Model System with Nitriles

Characteristic	N_2-ase	Model system with ATP
Products from unbranched aliphatic nitriles C_2–C_4	C_2–C_4 alkanes (NH_3)	C_2–C_4 alkanes (NH_3)
Apparent K_m values, mM (for CH_3CN)	~500	~1000
Rate rel to N_2 or C_2H_2 reduction	~0.5%	~0.01%
E_a (CH_3CN)[a]	14	13
Rate of CH_3CN reduction in D_2O rel to H_2O	2.5–3.5	2.6
Products from acrylonitrile	C_3H_6, C_3H_8 (6:1) (NH_3)	C_3H_6, C_3H_8 (3:1) (NH_3)
Apparent K_m, mM (for CH_2=CHCN)	10–50	~250
Rate rel to N_2 or C_2H_2 reduction	50%	0.02%
Products from crotononitrile	C_4H_8 isomers, C_4H_{10}	C_4H_8 isomers, C_4H_{10}
Apparent K_m (mM)	100–200 (cis isomer)	~100–150 (cis and trans isomers)
Rate rel to N_2 or C_2H_2	0.7% cis, 0.07% trans isomer	0.03 (cis and trans isomers)
Double bond shift during reduction of α-unsaturated nitriles	Observed	Observed
Substrate inhibition	Observed	Observed
Inhibition by CO and N_2	Observed, CO ≫ N_2	Observed, CO > N_2
Minimum requirements for reduction	N_2-ase, ATP, Mg^{2+}, $Na_2S_2O_4$	Complex I, $NaBH_4$, ($Na_2S_2O_4$), ATP[b,c]
Optimal pH	6–8	9–10
Effects of replacing Mo by V	Activity diminshes	Loss of activity
References	3,4	This work

[a] Temperature range between 15 and 40°. [b] Some reduction also occurs with MoO_4^{2-} in the absence of cysteine; reaction is very slow in the absence of substrate amounts of ATP; traces of Fe^{2+} salt have stimulating effect. [c] Reduction with $Na_2S_2O_4$ demonstrated with CH_3CN and CH_3CH=CHCN but only ~5% as efficient as $NaBH_4$.

Table IX. Sequence of Decreasing Rate of Reduction of Nitriles by Nitrogenase and the MoO_4^{2-}–Cys Model System

System	Decreasing Rate of Reduction
Nitrogenase	CH_2=CHCN > *cis*-CH_3CH=CHCN > CH_3CN > *trans*-CH_3CH=CHCN > CH_2=C(CH_3)CN ≥ C_2H_5CN
MoO_4^{2-}–Cys	*trans*-CH_3CH=CHCN > *cis*-CH_3CH=CHCN > CH_2=CHCN > CH_2=C(CH_3)CN > CH_3CN > C_2H_5CN

component. In the absence of ATP, for example, less *n*-butane and *cis*-2-butene are produced, while 1-butene and *trans*-2-butene are the principal products. With MoO_4^{2-}–GSH–ATP catalyst the rate of reduction of *cis*-crotononitrile relative to the trans isomer increases substantially. In addition, the yield of *cis*-2-butene increases from *cis*-crotononitrile as the substrate. In the reduction of *trans*-crotononitrile by MoO_4^{2-}–GSH–ATP the ratio of *trans*- to *cis*-2-butene is approximately 4:1, indicating that the cis–trans isomerization steps are impaired, conceivably due to a restriction of the rotation of the substrate residue in the intermediate organomolybdenum complexes. The effect is absent or not quite as noticeable with AET or BSA as the thiol component (Table VII).

Comparison with Nitrogenase Reactions. The main features of the enzymatic and nonenzymatic reduction of nitriles are summarized in Table VIII. The results clearly indicate that the N_2-ase reactions with nitriles are characteristic of a molybdenum active site, and that this site is reproduced to a good and valid approximation by MoO_4^{2-}–Cys and related molybdothiol catalysts. In accord with the results of the studies with acetylenes and isonitriles,[6,7] partial steric hindrance at the molybdenum-active site of N_2-ase is also noticeable with nitriles as substrates. In contrast, the MoO_4^{2-}–Cys model systems catalyze the reduction of straight chain as well as of branched aliphatic and olefinic nitriles, which are not reduced by N_2-ase.[3,4] In addition, the sequence of decreasing rate of reduction for a variety of nitriles differs as shown in Table IX. The observed double bond isomerization in the reduction of crotononitrile, which leads to the formation of 1-butene, is also gratifying. However, there are some minor differences between N_2-ase and the models. Thus, *cis*-crotononitrile is reduced more rapidly by N_2-ase than the trans isomer. The opposite is the case with MoO_4^{2-}–Cys catalysts. In addition, N_2-ase reduces *trans*-crotononitrile mainly to *trans*-2-butene, the cis isomer essentially to 1-butene and *n*-butane. The preference of the enzyme for *cis*-crotononitrile must be ascribed to a steric effect, limiting the accessibility of the molybdenum active center. The substrate, once attached to the molybdenum, could be held in a fixed position by the surrounding protein constituents. This is indicated by the fact that cis–trans isomerization, which should give rise to a mixture of *cis*- and *trans*-2-butene, is not observed with N_2-ase. Exchanging Cys as the thiol component by ligands such as bovine serum albumin (BSA) and glutathione (GSH), the cis–trans 2-butene ratio observed indicates that this isomerization is partially impaired. With MoO_4^{2-}–GSH the rate reduction of *cis*- and *trans*-crotononitrile becomes more nearly equal, thus indicating a move toward greater resemblance with the enzyme. Similar effects, though less pronounced, were observed with MoO_4^{2-}–BSA catalysts, indicating influences of the protein structure on the catalytic activity. The reduction of *cis*- and *trans*-crotononitrile by MoO_4^{2-}–Cys in the absence of ATP diminishes the relative yields of *n*-butane and of *cis*-2-butene, while producing *trans*-2-butene in comparable relative yields to 1-butene (Table VI). Considering that *trans*-2-butene is the principal product of enzymatic reduction of *trans*-crotononitrile it becomes plausible to assume that *trans*-crotononitrile, once attached to the molybdenum active site of N_2-ase, sterically prevents ATP in its approach to the molybdenum active site.

The function of iron in N_2-ase appears to be confined to electron activation and the catalysis of electron transport to the molybdenum active site. The molyb-

denum active site itself must be mononuclear. We stress this point in view of the occasional notion that the active site in N_2-ase either contains a binuclear molybdenum unit or consists of a Fe–Mo cluster-type complex. Our study of the model systems shows that binuclear molybdenum complexes are invariably either catalytically inactive or less active than monomeric species, and that the assumption of Fe–Mo clusters is unessential to account for the mechanism of N_2-ase action. The complete inactivity of VO_4^{3-}–Cys catalyst systems in the nitrile reduction parallels the results of recent work on vanadium–N_2-ase,[4] which reduces nitriles at only about 1–5% of the rate of molybdenum–N_2-ase. Conceivably, vanadium–N_2-ase owes its catalytic activity to the small residual amounts of molybdenum present in all preparations.

Whereas N_2-ase specifically requires substrate amounts of ATP for activity, the model systems are also stimulated by other phosphates, including GTP. Although we also noted a weak stimulation of catalytic activity by ADP and AMP, it appears that this is due to effects other than phosphorylation. The nature of the interactions of ATP and of the other phosphates with the catalyst systems will be discussed in a forthcoming paper.

Experimental Section

Reagents and Chemicals. Na_2MoO_4 (Baker analyzed Reagent), Na_3VO_4, and Na_2WO_4 (Alfa Inorganics) were recrystallized three times before use. $NaBH_4$ (Ventron) was recrystallized from diglyme according to published procedures.[12,13] L(+)-Cysteine·HCl, glutathione, bovine serum albumin, and all metal salts employed were used without further purification. The Mo(V) complex of cysteine, $Na_2Mo_2O_4(Cys)_2 \cdot 5H_2O$, was prepared according to Kay and Mitchell.[14] Cylinder nitrogen was 99.998%; argon, 99.995% (both from National Cylinder Gas), and carbon monoxide C. P. (Matheson) was 99.9%. All gases were passed through alkaline pyrogallol solution and water before entering the reaction vials. All nitriles were commercial grade products and were fractionally distilled before use. Crotononitrile (Eastman) consisted of a mixture of the cis and trans isomer. Separation of the isomers was achieved through fractional distillation using a spinning band column.[15]

Standard Gas Chromatographic Technique. All hydrocarbons were determined by glpc; experimental details are given in ref 7.

Standard Experimental Procedures. Procedures employed in the present paper are essentially identical with those described in ref 7; further details are outlined in the legends to the figures and tables. A typical procedure for catalytic nitrile reduction is given below.

Catalytic Nitrile Reduction. A 1.25×10^{-4} *M* stock solution of complex I was prepared by dissolving 6.3 mg of crystalline "complex I" in 80 ml of deoxygenated, 0.2 *M* pH 9.6 borate buffer. The solution was purged with argon for 15 min and stored in a 100-ml glass bottle fitted with a rubber septum. Aliquots of this solution (*e.g.*, 1.0 ml) were injected into screw-cap rubber septum vials of 25 ml volume (available from Precision Sampling Corp., Baton Rouge, La. 70815, Catalogue No. 630063), which were previously purged with argon. The volume of the complex I solution was subsequently brought to a total of 3.0 ml by adding 0.2 *M* pH 9.6 borate buffer. The nitrile substrate was added next by means of a syringe, usually in amounts of about 0.1 ml. At $t = 0$, 0.5 ml of a freshly prepared 1.33 *M* $NaBH_4$ solution in 0.2 *M* pH 9.6 borate buffer was injected to initiate the reaction. In the experiments run with ATP, a specified amount (usually 0.5 ml) of a freshly prepared 1.2 *F* ATP solution in 0.2 *M* pH 9.6 borate buffer was injected immediately following the addition of the $NaBH_4$ solution. The vials were gently shaken and were maintained at 25° during the experiment. At specified time invervals (*e.g.*, 10, 15, or 20 min after the addition of $NaBH_4$ or ATP) the gas pressure in the vials was first reduced to 1 atm by allowing the gas (mostly hydrogen) to expand into an empty syringe of volume of 60 ml. For glpc measurements, 0.2-ml samples were withdrawn by means of a small gas syringe. To continue the experiment the excess gas was pushed back into the vial.

Inhibition by CO and N_2. The inhibition of nitrile reduction by CO and N_2 was studied as described above except that the vials were previously purged with the inhibitor gases at 1 atm of pressure. In all cases parallel experiments were carried out under 1 atm of argon.

Nitrile Reduction with Systems Containing Other Metals. Stock solutions of metal salts (mostly of the chlorides, 0.0042 *M*) and of Cys·HCl (0.0042 *M*) in buffer were prepared and stored under argon. Reaction vials were first purged with argon. Subsequently, 1.5 ml of each of the two stock solutions was injected, followed by 0.2 ml of CH_3CN substrate. After 5 min of incubation at room temperature, the reaction was initiated by injecting 0.5 ml of fresh 1.33 *M* $NaBH_4$, and where indicated, 0.5 ml of fresh 1.2 *M* ATP solution (both in 0.2 *M* pH 9.6 borate buffer). Sample withdrawal and inhibition experiments with CO and N_2 were conducted as described above.

K_m Values. The apparent K_m values were obtained from Lineweaver–Burk plots from initial rate data at various substrate concentrations.

Experiments with $^{15}N_2$. To demonstrate the reduction of molecular nitrogen under the conditions of nitrile reduction $^{15}N_2$ was used. Screw-cap, rubber septum vials of the type described above were first filled with 1 atm of 99 atom % $^{15}N_2$. Subsequently, 3.0 ml of freshly prepared 0.05 *M* $Na_2Mo_2O_4(Cys)_2 \cdot 5H_2O$ in 0.2 *M* pH 9.6 borate buffer was injected, followed by 0.5 ml of 0.35 *M* CH_3CN solution. The reaction was initiated by injecting 0.5 ml of freshly prepared 1.33 *M* $NaBH_4$ in pH 9.6 buffer and 0.5 ml of freshly prepared 1.2 *M* ATP solution, both in pH 9.6 borate buffer (0.2 *M*). After standing for 2 days at 27°, the reaction solutions were acidified with 0.2 ml of 12 *N* HCl, and quantitatively transferred into one of the limbs of a Rittenberg apparatus,[16] and oxidized by freshly prepared hypobromite as described in ref 16. The nitrogen evolved was analyzed mass spectroscopically. Since a significant part of the nitrogen formed arises from NH_3 produced through nitrile hydrolysis, the nitrogen produced was predominantly $^{14}N_2$ and $^{14}N \equiv {}^{15}N$. After correction for natural abundance, the calculated amount of $^{15}N_2$ fixed was 0.22 μmol. In the absence of CH_3CN under otherwise identical conditions an amount equivalent to 0.58 μmol of NH_3 formed from $^{15}N_2$ was reproducibly observed.

Acknowledgments. This work was supported by Grant No. GP28485X of the National Science Foundation and, in part, by a grant from Climax Molybdenum Co., a subsidiary of American Metal Climax, Inc.

(12) H. C. Brown, *et al.*, *J. Amer. Chem. Soc.*, 77, 6209 (1955).

(13) The recrystallized $NaBH_4$ contains some diglyme of crystallization which does not interfere with the nitrile reduction reactions. Interference by ethylene glycol was noted, however, giving rise to ethylene. Ethylene glycol should therefore not be used to improve the solubility of water-insoluble substrates for studies with our catalyst systems.

(14) A. Kay and P. C. H. Mitchell, *Nature (London)*, 219, 267 (1967).

(15) We also thank Drs. R. W. F. Hardy and R. Burns for supplying samples of the two isomers.

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