Chemical Evolution of a Nitrogenase Model. VI. The Reduction of CN^-, N_3^-, N_2O, N_2, and Other Substrates by Molybdocysteine Catalysts in the Presence of Nucleoside Phosphates[1]

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Abstract: Nitrogenase model systems composed of molybdate and sulfur containing ligands such as L(+)-cysteine catalyze the reduction of CN^-, N_3^-, N_2O, and N_2 with $NaBH_4$ as the reductant. All reactions are stimulated by ATP and other nucleoside phosphates. The reduction of CN^- yields CH_4, NH_3, smaller amounts of C_2H_6, C_2H_4, and traces of CH_3NH_2; N_3^- and N_2O are reduced to N_2 and NH_3 and N_2 and H_2O, respectively, as with nitrogenase. The molybdate–cysteine catalysts also reduce ethylene oxide to C_2H_4 and H_2O and ethyl diazoacetate to ethyl acetate plus N_2; these reductions are also stimulated by ATP. In all reactions, added iron salts act as cocatalysts. Experiments with $^{15}N_2$ indicate that the fixation of N_2 is molybdenum-specific. A comparison of the relative rates of reduction of all substrates indicates parallels with the relative rates of reduction observed with nitrogenase. The K_m values for reactions of substrates in the model system are as a rule larger than those in the enzymatic reduction, indicating a weaker binding of the substrates by the nonenzymatic catalysts. A detailed investigation of the effect of ATP and of other nucleoside phosphates reveals that two molecules of the phosphates interact with the catalyst and that ATP hydrolysis to ADP and inorganic phosphate is unrelated to the actual stimulatory effect. The stimulation of catalytic activity is higher in borate than in phosphate buffer; in the former, addition of Mg^{2+} has no effect; in the latter, Mg^{2+} ion increases the rate of CN^- reduction at certain concentrations presumably by binding the inhibitory phosphate ion.

In the previous five papers of this series[3-7] we reported on the reduction of nitrogenase (N_2-ase) substrates in nonenzymatic model systems composed of molybdate and a thiol (usually L(+)-cysteine, Cys), with $NaBH_4$ or $Na_2S_2O_4$ as reductants, and demonstrated the exceptional abilities of these model systems to duplicate reactions typical of N_2-ase holoenzyme. In this paper we describe similar studies with other alternate substrates of N_2-ase,[8] *i.e.*, with CN^-, N_3^-, and N_2O. Results with N_2 are also quoted but only to permit comparisons with the behavior of the other substrates; the mechanism of N_2 reduction in our catalyst system will be outlined in greater detail in a forthcoming publication. Brief mention will also be made of the reduction of $N_2CHCOOC_2H_5$ (ethyl diazoacetate) and of C_2H_4O (ethylene oxide). The results of extensive studies concerning the effects of ATP and of other nucleoside phosphates and of Mg^{2+} ion in our system will be described as well.

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Results

Model Systems Employed. As in our previous work,[3-7] the reductions of the substrates to be reported here were usually performed using the binuclear Mo(V)–Cys complex (complex I) as the catalyst. This complex is not the actually active form of the catalyst, which is a monomeric species. Under the reaction conditions, complex I dissociates into monomeric Mo-Cys complexes which are converted into the active reduced forms in the presence of reducing agents. Complex I can be replaced by 1:1 mixtures of MoO_4^{2-} and Cys. These were employed whenever this proved necessary or advantageous. The reaction conditions chosen were identical with those employed previously and are essentially optimal. The solutions were buffered to the pH of approximately 9.6. Borate buffer and, in some experiments, phosphate buffer were used at concentrations of 0.2 *F*. The reducing agent was $NaBH_4$; the reaction temperature was 27° unless specified. ATP and other nucleoside phosphates were used as additional cofactors, both in the presence and absence of Fe^{2+} (supplied as $FeSO_4 \cdot 7H_2O$) as the cocatalyst.

Characteristics of the Reduction of CN^-, N_3^-, N_2O, and N_2. The highest catalytic activity for the reduction of the substrates of the present study was observed with systems containing MoO_4^{2-}, Cys, Fe^{2+}, and substrate amounts of ATP, with $NaBH_4$ as the reductant in borate buffer. The effects of omission of any one or more of these systems components are summarized in Table I. It may be seen that Fe^{2+} omission causes only a relatively small diminution of catalytic activity, except with N_2 as the substrate, where omission of Fe^{2+}

Table I. Relative Rates of Product Formation from CN^-, N_3^-, N_2O, and N_2[a] in pH 9.6, 0.2 *F* Borate Buffer at 27°

No.	System	Relative rates of product formation[b] CN^-	N_3^-	N_2O	N_2[c]
1	Complete	100	100	100	100
2	1, $-Fe^{2+}$	83	70	85	62
3	2, −ATP	14	0.2	~0.1	50
4	3, +hydrolyzed ATP	17	0.2	~0.2	43
5	2, $-MoO_4^{2-}$	0	0	0	0
6	2, −Cys	5	0.1	0.5	7.7
7	2, $-BH_4^-$	0	0	0	0
8	2, −substrates	0	0.1	0	0
9	−, $+BH_4^-$, +ATP	4	0.1	0.1	0
10	−, $+BH_4$	4	0.2	0.1	0

[a] At approximately optimal catalyst and substrate concentrations for the complete systems: $[CN^-]_{initial}$ = 0.025 *M* (MoO_4^{2-} = Cys = 0.0083 *M*); $[N_3^-]_{initial}$ = 2.5 *M* (MoO_4^{2-} = Cys = 0.0037 *M*); $[N_2O]_{initial,\ gas\ phase}$ = 0.36 atm (MoO_4^{2-} = Cys = 0.0075 *M*); $[N_2]_{initial,\ gas\ phase}$ = 1 atm (complex I = 0.0050 *M*); $[Fe^{2+}] = 2 \times 10^{-4}$ *M* (supplied as $FeSO_4 \cdot 7H_2O$); $[ATP]_{initial}$ = 0.075–0.15 *M*. Total reaction volume is 4–6 ml; volume of reaction vials is 30 ml. [b] Initial absolute rates of complete systems: 0.568 nmol/min (for CN^-), 0.045 μmol/min (for N_3^-), 0.241 μmol/min (for N_2O), 0.11 μmol/min (for N_2), all at 27°. Rates were determined by monitoring the amount of CH_4 (from CN^-), NH_3 (from N_3^-), and N_2 (from N_2O) and by mass spectrographic determination of ^{15}N from OBr^- oxidized reaction solutions (for N_2). [c] Rates are based on the total yield of ^{15}N released from the reaction solutions on OBr^- oxidation (corrected for contamination by ^{15}N arising from unlabeled nitrogen or nitrogen compounds present). The rates thus reflect the formation of NH_3 as well as of precursors of NH_3 (N_2H_2 and N_2H_4) in these systems.

diminishes nitrogen fixation to 62% as compared with the complete system. The Fe^{2+} functions as a nonspecific electron transfer catalyst,[3-7] however, and for this reason was usually omitted to maintain the number of systems components as small as possible. Iron, in the absence of molybdenum and in homogeneous solution, has either no or at best minor catalytic activity. Its addition to the molybdo–Cys catalyst system does not lead to the formation of stable Fe–Mo–Cys cluster complexes or defined sulfur-bridged Fe–Mo species, since the overall behavior of the catalysts as judged from the observed product distribution from various substrates remains virtually unchanged as compared with the systems containing only molybdenum and Cys. The data compiled in Table I thus demonstrate conclusively that the omission of any of the components of the complete system has similar effects as described in all of our previous studies. The results of representative rate measurements with the substrates of the present study (excluding N_2) are summarized in Tables II and III. Typical product yield–time curves are shown in Figure 1.

Reduction of CN^-. The reduction of CN^- by the complete system in the absence of ATP is extremely slow (see Figure 2), yielding mainly CH_4 and traces of C_2H_6 and C_2H_4. The addition of freshly prepared ATP solution causes an increase in the hydrocarbon production which is initially proportional to $[ATP]^2$. GTP is equally as active a stimulant as ATP. ADP and AMP are less effective, but the stimulation of hydrocarbon

Table II. Rates and Product Ratios of the Complex I Catalyzed Reduction of CN^- under Various Conditions

System and reaction conditions[a]				Initial rate[c]	Product ratios
$[CN^-]$	[Catalyst]	[Nucleoside phosphate]	Gas phase, pH, buffer, cofactor, and solvent[b]	(nmol/min)	$CH_4/C_2H_6/C_2H_4$
0.025	0.0083 MoO_4^{2-}–Cys	...	Ar, 9.6, B, ..., H_2O	0.009	100/trace/trace
		0.075 ATP		0.472	100/21/81
0.025	0.0188 complex I	...		0.014	100/trace/trace
		0.075 ATP or GTP		1.05	100/24/24
		0.022 ATP		0.045	100/0/12
		0.022 ADP		0.036	100/0/10
		0.022 AMP		0.028	100/0/10
		0.022 adenine		0.014	100/trace/trace
		0.022 adenosine		0.013	100/trace/trace
		0.022 $Na_5P_3O_{10}$		0.013	100/trace/trace
		0.022 $Na_4P_2O_7$		0.014	100/trace/trace
		...	Ar, 9.6, P, ..., H_2O	0.003	100/trace/trace
		0.022 ATP		0.147	100/20/40
		0.022 ADP		0.033	100/1/1
		0.022 AMP		0.070	100/trace/24
		0.075 ATP	N_2, 9.6, B, ..., H_2O	0.55	100/24/21
			CO, 9.6, B, ..., H_2O	0.68	100/18/15
			O_2, 9.6, B, ..., H_2O	0.85	100/20/21
			Ar, 7.0, P, ..., H_2O	0.091	100/0/4
			Ar, 8.0, P, ..., H_2O	0.103	100/0/4
			Ar, 9.0, P, ..., H_2O	0.128	100/0/4
			Ar, 10.0, P, ..., H_2O	0.108	100/0/4
			Ar, 11.0, P, ..., H_2O	0.100	100/0/4
			Ar, 9.6, B, 0.006	Fe^{2+}, H_2O 1.26	100/4/6
			Ar, 9.6, B, 0.025	Mg^{2+}, H_2O 1.04	100/21/24
			Ar, 9.6, P, 0.025	Mg^{2+}, H_2O 1.45	100/15/54
			Ar, 9.6, P, 0.075	Mg^{2+}, H_2O 0.75	100/0/31
			Ar, 9.6, P, 0.127	Mg^{2+}, H_2O 1.15	100/16/8
		...	Ar, 9.6, B, ..., D_2O	0.021	100/trace/trace
		0.075 ATP	Ar, 9.6, B, ..., D_2O	2.41	100/10/14
		0.125 ATP	Ar, 9.6, B, ..., H_2O	2.75	100/23/25

[a] All concentrations in units mol/l. of the reaction solution. [b] Gas phase denotes the gas initially present in reaction vial, all at 1 atm. B = borate, P = phosphate buffer, both 0.2 *F*. [c] Rate of CH_4 production.

Table III. Rates and Product Ratios of the Complex I Catalyzed Reduction of N_3^-, N_2O, $N_2CHCO_2C_2H_5$, and C_2H_4O (ethylene oxide)

[Substrate][a]	[Catalyst]	[Nucleoside phosphate]	Gas phase,[b] pH, buffer	Initial rate	Products[c]
N_3^-					
2.50	0.0037 MoO_4^{2-}–Cys	...	Ar, 9.6, B	0 μmol/min	N_2, *NH_3*
		0.15 ATP	Ar, 9.6, B	0.032	
1.67	0.002 complex I	...	Ar, 9.6, B	0	
		0.125 ATP	Ar, 9.6, B	0.059	
0.0				0	
0.16				0.013	
0.50				0.028	
5.00				0.013	
	...			0	
	0.021			0.034	
	0.066			0.020	
1.67	0.002	0.125 ADP		0.046	
		0.125 AMP		0.022	
		0.125 adenine		0	
		0.125 $Na_5P_3O_{10}$		0.001	
		0.125 ATP	Ar, 8.0, P	0.031	
			Ar, 9.0, P	0.048	
			Ar, 10.0, P	0.032	
			Ar, 11.0, P	0.010	
			CO, 9.6, B	0.032	
N_2O					
0.36 atm	0.0075 MoO_4^{2-}–Cys	...	Ar, 9.6, B	0.001	N_2, H_2O
		0.15 ATP	Ar, 9.6, B	0.202	
	0.019 complex I	...		0	
		0.15 ATP		0.172	
...				0	
0.10				0.057	
0.50				0.173	
1.00			(N_2O), 9.6, B	0.182	
0.36	...		Ar, 9.6, B	0	
	0.010 complex I			0.083	
	0.075 complex I			0.069	
	0.019 complex I	0.15 ADP		0.141	
		0.15 AMP		0.043	
		0.15 adenine		0	
		0.15 adenosine		0.004	
		0.15 $Na_5P_3O_{10}$		0	
		0.15 ATP	CO, 9.6, B	0.095	
$N_2CHCO_2C_2H_5$					
0.019	0.018 complex I	...	Ar, 9.6, B	7.01 nmol/min	*$CH_3COOC_2H_5$*,[d] N_2
		0.15 ATP		14.30	
			Ar, 7.0, P	5.79	
			Ar, 9.0, P	7.57	
	...		Ar, 9.6, B	0	
	0.0007 complex I			10.9	
	0.18			10.0	
...	0.018			0	
0.009				1.50	
0.030				9.30	
C_2H_4O					
...	0.00	...		0	*C_2H_4*, H_2O
1.00	0.019 complex I	...		0.56	
		0.15 ATP		0.93	

[a] All concentrations in units mol/l. of the reaction solution. [b] Gas phase denotes the gas initially present in reaction vial, all at 1 atm. B = borate, P = phosphate buffer. [c] Assayed product italicized. [d] Ethyl acetate is formed first; yields are corrected for saponification of product at prolonged reaction times.

production by these nucleoside phosphates is initially also proportional to [nucleoside phosphate]^2 (Figure 3). Higher concentrations of nucleoside phosphates eventually become inhibitory, as will be outlined below. In addition to the aforementioned products, traces of CH_3NH_2 are formed in the ATP stimulated reactions. The overall reaction may thus be represented according to eq 1. The ratio of $CH_4:C_2H_6:C_2H_4$ depends on

$$CN^- + 6\text{–}12e \xrightarrow[H_2O]{\text{Mo–cys catalyst, ATP } (Fe^{2+})} CH_4, C_2H_6, C_2H_4, CH_3NH_2, NH_3 \quad (1)$$

several factors, including the initial concentrations of substrate, reductant, and ATP. Under optimal conditions it is about 1:0.2:0.2. Rate data and product ratios under various conditions are summarized in Table II.

The reduction of CN^- is inhibited not only by high concentrations of substrate but also by O_2, N_2, and CO relative to runs under argon as protective gas. At 1 atm of partial pressure, O_2 causes a 20% inhibition of CH_4 and C_2 hydrocarbon production. Most interestingly, N_2 is a stronger inhibitor than O_2 under the same conditions, lowering the yield of CH_4 to 56% as compared with runs under argon. The nitrogen is reduced to NH_3 even in the presence of CN^-, as was demonstrated

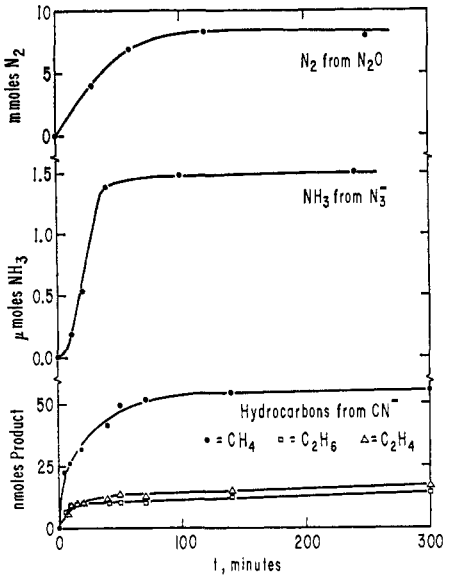


Figure 1. Catalytic reduction of CN^-, N_3^-, and N_2O by complex I-$NaBH_4$ in the presence of substrate amounts of ATP, during the first 300 min of reaction at 27°. Initial concentrations: $[CN^-]$ = 0.025 *M* (complex I, 0.019 *M*, $NaBH_4$, 0.165 *M*); $[N_3^-]$ = [ATP] = 0.125 *M* (complex I, 0.0021 *M;* $NaBH_4$, 0.11 *M*). $[N_2O]$ = 0.39 atm (complex I, 0.0019 *M;* ATP, 0.15 *M;* $NaBH_4$, 0.165 *M*). All in pH 9.6 and 0.2 *F* borate buffer (total reaction volume 4–6 ml).

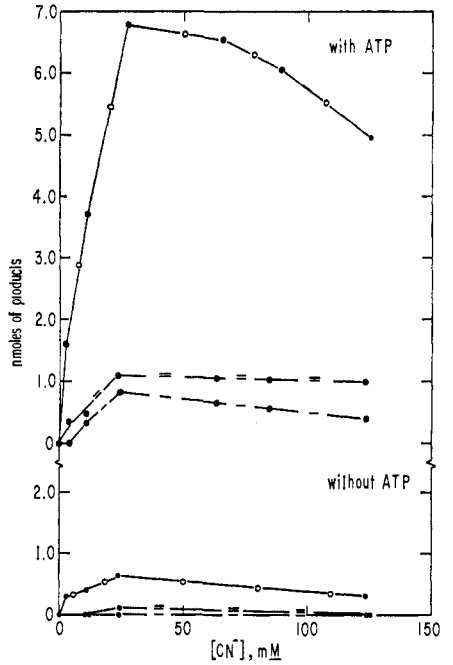


Figure 2. Dependence of product yields (CH_4, —○—○—; C_2H_6, -═-═; C_2H_4, - - - - -) as a function of $[CN^-]$, after 30 min of reaction, in the presence and absence of ATP. Initial concentrations: complex I, 0.019 *M;* $NaBH_4$, 0.165 *M;* [ATP], 0.075 *M*. Total reaction volume 4 ml, in pH 9.6 and 0.2 *F* borate buffer.

with $^{15}N_2$. Inhibition of CN^- reduction by CO was also observed. However, CO is reduced to CH_4 in our system if BH_4^- is employed as the reducing agent (the initial rate of CH_4 production at 1 atm of CO is 4.1 nmol/min at the experimental conditions given in Table II). Correcting the yield of CH_4 for the amount arising from CO reduction, the conversion of CN^- to CH_4 is 65% of that observed under argon. The

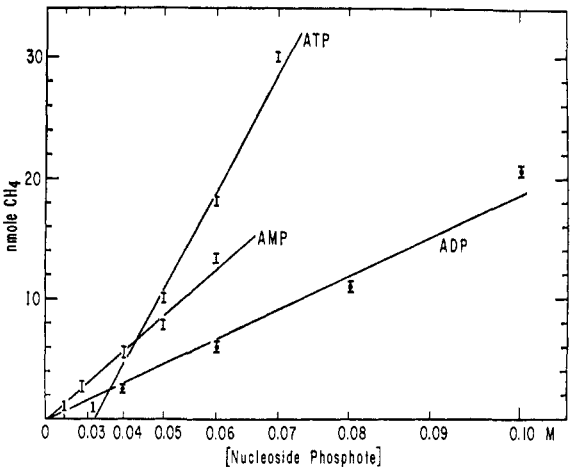


Figure 3. Dependence of product yields in CN^- reduction on $[ATP]^2$, $[ADP]^2$, and $[AMP]^2$. Abscissa denotes [nucleoside phosphate] on a parabolic scale. Initial concentrations: complex I, 0.019 *M;* $NaBH_4$, 0.165 *M;* $[CN^-]$, 0.025 *M*. Total reaction volume 4 ml, in pH 9.6 and 0.2 *F* borate buffer.

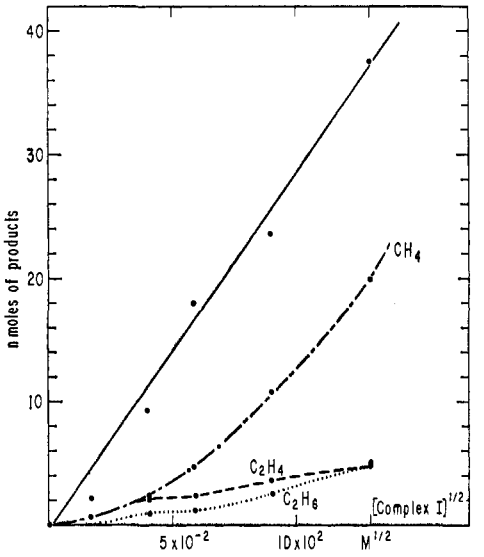


Figure 4. Product yields at low catalyst concentrations as a function of $[complex]^{1/2}$ (for experimental conditions see caption of Figure 5). The straight line represents the total hydrocarbon production ($CH_4 + \frac{1}{2}C_2H_4 + \frac{1}{2}C_2H_6$).

corrected $CH_4:C_2H_6:C_2H_4$ ratio is 1:0.18:0.15. It thus is not substantially altered by CO, indicating that CO does not interfere with the C_2 hydrocarbon production.

The time dependence of hydrocarbon formation from CN^- in the ATP stimulated reaction is nonlinear presumably owing to the concomitant hydrolysis of ATP to ADP and inorganic phosphate (P_i) and the loss of reducing power owing to decomposition of BH_4^- (Figure 1). At concentrations of catalyst of up to 0.018 *M* there is a linear dependence of the total hydrocarbon production as a function of $[complex\ I]^{1/2}$, providing conclusive evidence for the presence of a mononuclear Mo–Cys species as the catalyst (Figure 4). A further increase of the concentration of complex I causes an exponential decline of catalytic activity (Figure 5), indicating that the preequilibria leading to mononuclear derivatives are no longer established rapidly. Conversely, if complex I is substituted by equimolar mixtures of MoO_4^{2-} and Cys, the catalytic activity is greater

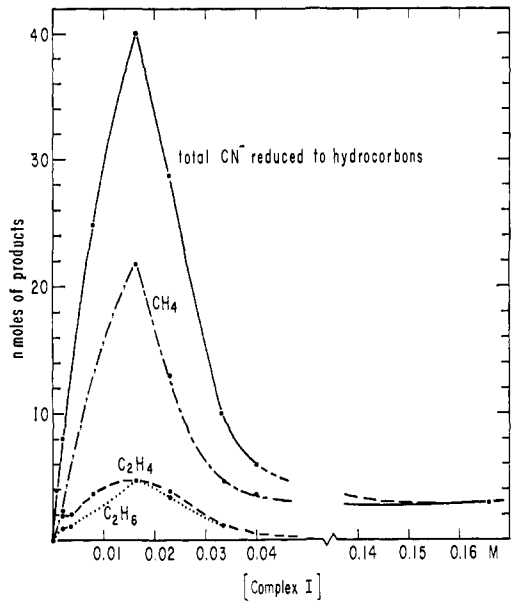


Figure 5. Product yields from CN^- as a function of [complex I]. Yields of CH_4, ·-·-·; C_2H_4, – – –; C_2H_6, ...; and total hydrocarbons ($CH_4 + 2C_2H_4 + 2C_2H_6$) after 30 min of reaction. Initial concentrations: $[CN^-] = 0.075$ *M*, all others as in caption of Figure 2.

since the formation of inactive binuclear species is a relatively slow process.

The dependence of the rate of hydrocarbon production on $[CN^-]$ is approximately linear up to $[CN^-]_{initial} = 0.025$ *M;* at higher concentrations substrate inhibition is observed (Figure 2). The K_m value for CN^-, determined from Lineweaver–Burk plots, is 12 m*M* at 27°. Owing to the volatility and instabilityof HCN at elevated temperatures, it is difficult to obtain reliable estimates of the Arrhenius energy of activation (it appears to be in the order of 12 kcal between 20 and 30°). Exchanging H_2O for D_2O as the reaction medium causes a stimulation of hydrocarbon production by a factor of 2.3 and a diminution of the relative yield of C_2 hydrocarbons (Table II).

Reduction of Ethylene Oxide. The reduction of ethylene oxide with our catalyst system was investigated to explain the mechanism of C_2H_4 formation from CN^- (see Discussion Section). Ethylene oxide is reduced to C_2H_4 by complex I–$NaBH_4$; the reaction is ATP stimulated and proceeds according to the summary

$$\underset{\diagdown\,\diagup}{\overset{O}{}}\!\!CH_2CH_2 + 2e + 2H^+ \xrightarrow{\text{Mo–Cys catalyst, ATP}} C_2H_4 + H_2O \quad (2)$$

Rate data are summarized in Table III. Ethylene oxide as a possible alternate substrate of N_2-ase has not yet been studied in detail. Traces of ethylene were detected after incubation of cell extracts of *Azotobacter chroococcum* with ethylene oxide. The reaction does not appear to be genuinely catalytic, however (unpublished experiments by Dr. J. R. Benemann).

Mechanism of Nucleoside Phosphate Action. The effects of ATP and of other nucleoside phosphates were investigated using CN^- as the substrate probe for catalytic activity of complex I–BH_4^- systems. ATP accelerates the conversion of oxidized forms of the molybdo–Cys catalyst to the active reduced form, but the manner in which ATP actually functions has not yet been specified. Since ATP could in principle act as a phosphorylating agent for inert molybdenum-bound OH^- groups, we have first investigated the effect of MoO_4^{2-} as well as of I on the rate of ATP hydrolysis under the experimental conditions used for substrate reduction. Using ^{14}C labeled ATP it was observed that both MoO_4^{2-} and I catalyze the hydrolysis of ATP to ADP and P_i (Table IV), both in the absence and presence of reductant (BH_4^-).[9] Analogous experiments with ^{14}C labeled ADP and AMP revealed that their hydrolysis to either AMP or adenosine and P_i does not occur in the presence or absence of MoO_4^{2-} or complex I. Since both ADP and AMP stimulate the catalytic activity, however, this indicates that these nucleoside phosphates are functional even though they are not phosphorylating agents in the classical sense. A more detailed study of the effects of ATP, ADP, and AMP in our catalyst system was therefore necessary and is described in the following. It was first observed that the stimulatory effects of all nucleoside phosphates are significantly diminished in phosphate buffer, except at nucleoside phosphate concentrations of up to 0.05 *M* where rates in phosphate buffer are slightly greater than in borate (see Figures 6 and 7). Most measurements were for this reason performed in borate as well as in phosphate buffered solutions of the same pH. The results in Figures 6 and 7 indicate a similarity in the shapes of all [hydrocarbon]–[nucleoside phosphate] functions, except that the maxima occur at different concentrations. The [ATP]–[CH_4] function (Figure 6) in borate buffer shows a shoulder at high ATP concentrations which is most likely caused by the presence of ADP, the hydrolysis product formed under the reaction conditions, since it occurs at the same concentrations at which ADP exhibits maximal efficiency. ADP is less active at concentrations which are optimal for ATP,

Table IV. Effect of MoO_4^{2-} and of Complex I on the Hydrolysis of ATP, ADP, and AMP in the Presence and Absence of CN^- and of Reductant ($NaBH_4$)[a] (Experiments in collaboration with R. H. Frazier, Jr.)

Nucleoside phosphate	System[b]	Concn of catalyst (Mo, *M*)	% radioactivity ATP	ADP	AMP	Unknown spots
ATP	Blank, $t = 0$	0	98	1	1	
	Blank, BH_4^-	0	90	8	1	
	I, BH_4^-, CN^-	0.005	89	9	0.8	
	I, BH_4^-, CN^-	0.01	88	10	0.7	Trace
	I, BH_4^-, CN^-	0.025	87	11	0.8	Trace
	I	0.025	89	10	0.8	Trace
	MoO_4^{2-}	0.025	85	13	1.6	
	I, BH_4^-, CN^-	0.050	83	16	1	
ADP	Blank, $t = 0$	0	0.4	95	4	0
	I, BH_4^-, CN^-	0.025	0.6	92	4	3
AMP	Blank, $t = 0$	0	0	0	100	0
	I, BH_4^-, CN^-	0.025	0	0	98	2

[a] In borate buffer (pH 9.6, 0.2 *F*). Experiments were performed with ^{14}C labeled nucleoside phosphates; the identification of products was effected after addition of unlabeled ATP, ADP, and AMP prior to separation by paper chromatography. [b] Measurements performed after 15 min of reaction at 27° unless indicated differently.

(9) In some experiments ATP hydrolysis catalyzed by complex I was enhanced in the presence of BH_4^- as reducing agent, suggesting that complex I catalyzed ATP hydrolysis is further stimulated in the presence of reductant.

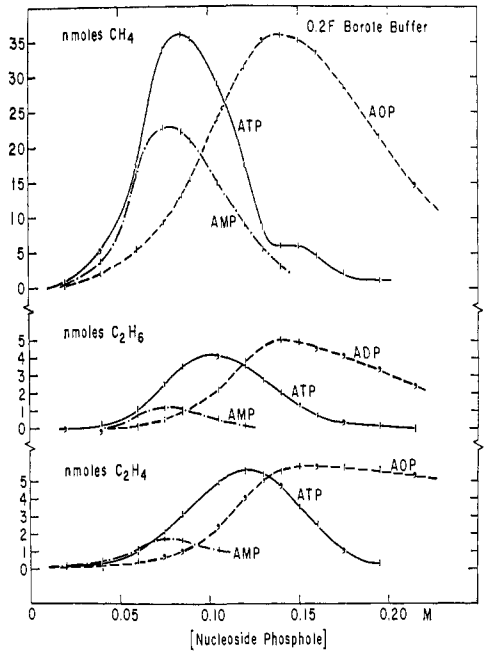


Figure 6. Dependence of product yields in the reduction of CN^- by complex I-$NaBH_4$ as a function of [nucleoside phosphate]. Initial concentrations as in Figure 3, in pH 9.6, 0.2 *F borate buffer*.

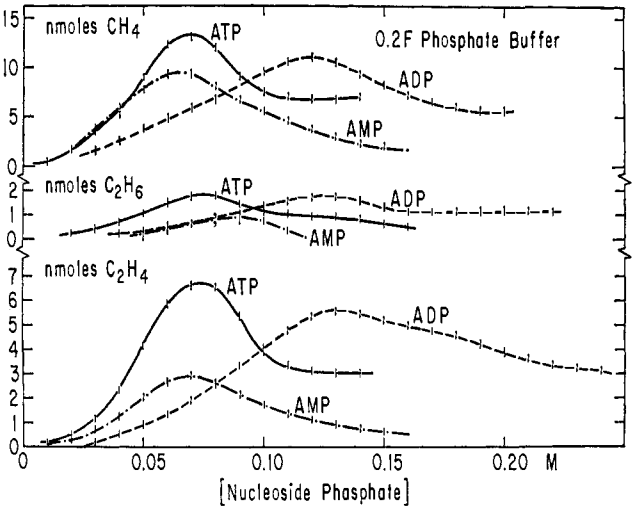


Figure 7. Dependence of product yields in the reduction of CN^- by complex I-$NaBH_4$ as a function of [nucleoside phosphate]. Conditions as in Figures 3 and 6, except that 0.2 *F*, pH 9.6, *phosphate buffer* was used.

but reaches an almost equal degree of efficiency at concentrations of around 0.14 *M* (Figure 6). AMP in borate buffer has only two-thirds of the efficiency of either ATP or ADP at optimal concentrations. The effects of ATP, ADP, and AMP in phosphate buffer at optimal concentrations are only about one-third to one-half those in borate buffer. Neither $P_2O_7^{4-}$, $P_3O_{10}^{5-}$, adenine, nor adenosine stimulate CH_4 production from CN^- in our system. For C_2H_6 and C_2H_4, maximal production occurs at somewhat higher concentrations of the nucleoside phosphates than for CH_4 in borate buffer. In phosphate buffer, the C_2 hydrocarbon yield maxima coincide with those observed for CH_4; C_2 hydrocarbon production is furthermore enhanced in phosphate relative to borate buffer. The fact that boiled ATP solutions have only approximately one-fifth of the stimulatory activity of fresh ATP (see Table I) may now be plausibly explained; the boiled ATP solutions contain ADP and P_i.[10] The former is less active than ATP at the same concentrations, while the latter (P_i) introduces an additional inhibitory effect.

The Effect of Magnesium Ion. Since N_2-ase requires the presence of Mg^{2+} ion for maximum activity,[11] its effect in the model system was also investigated. In borate buffer no appreciable enhancement of CN^- reduction was observed (see Table II). In phosphate buffer, Mg^{2+} ion is stimulatory. At low concentrations (*e.g.*, 0.025 *M*) the yields of CH_4, C_2H_6, and C_2H_4 are approximately 130% of those in the absence of Mg^{2+} under otherwise identical conditions. At high concentrations of Mg^{2+} (*e.g.*, 0.125 *M*) there is still a slight increase of CH_4 production, but the yields of C_2H_6 and C_2H_4 are nearly doubled. At intermediate Mg^{2+} levels the formation of all hydrocarbon products is diminished, suggesting that Mg^{2+} under certain conditions may also exert inhibitory effects (Table II). It should be emphasized, however, that all the results quoted here were observed in homogeneous solutions. Although the addition of Mg^{2+} (in form of $MgSO_4 \cdot 7H_2O$) to solutions of complex I and $NaBH_4$ in phosphate buffer causes the formation of a precipitate of magnesium phosphates, clear solutions invariably resulted as soon as ATP was added. It thus is certain that Mg^{2+} ion increases the efficiency of the model system. It is difficult, however, to decide if the observed Mg^{2+} effect is in any way related to that in N_2-ase, where this ion could also play the role of an allosteric effector.[12,13] In the model system, Mg^{2+} presumably functions by binding inorganic phosphate, thus diminishing its inhibitory effect.

Reduction of N_3^-, N_2O, and of $N_2CHCO_2C_2H_5$. The reduction of these three substrates is again very slow in the absence of ATP or other nucleoside phosphates. In the presence of substrate amounts of, *e.g.*, ATP, NH_3 and N_2 are formed from N_3^- (Figure 1). The overall reaction is given in eq 3.

$$N_3^- + 2e + 3H^+ \xrightarrow{\text{Mo-Cys catalyst, ATP}} NH_3 + N_2 \qquad (3)$$

The dependence of the rate of N_3^- reduction on the catalyst concentration is similar to that shown in Figure 5, except that the maximum catalytic activity is observed at concentrations of about 1.25×10^{-2} *M* of complex I. The dependence of the rate of N_3^- reduction on $[N_3^-]$ is linear only up to 0.01 *M;* higher substrate levels are inhibitory. The K_m value, determined from Lineweaver–Burk plots, is 0.5 ± 0.1 m*M* at 27°. At 1 atm of partial pressure, under conditions given in Table III, CO inhibits N_3^- reduction by 46%. The apparent Arrhenius energy of activation is 12 kcal between 20 and 37°. Rate data obtained for typical experiments are summarized in Table III.

The reduction of N_2O to N_2 and H_2O was in most experiments followed by mass spectroscopic determinations of the N_2 produced (for control purposes also by

(10) Identified by paper chromatography.
(11) T. O. Munson, M. J. Dilworth, and R. H. Burris, *Biochim. Biophys. Acta*, **104**, 278 (1965).
(12) The requirement for Mg^{2+} is nonspecific; also active are Mn^{2+}, Co^{2+}, Fe^{2+}, and Ni^{2+} (order of decreasing effectiveness).[13]
(13) R. C. Burns, *Biochim. Biophys. Acta*, **171**, 253 (1969).

measuring the decline of the initial N_2O concentration in the gas phase). The overall reaction is

$$N_2O + 2e + 2H^+ \xrightarrow{\text{Mo–Cys catalyst ATP}} N_2 + H_2O \quad (4)$$

The rate of N_2O reduction increases linearly with the substrate concentrations up to a partial pressure of 0.5 atm and subsequently levels off, suggesting substrate inhibition. The K_m is approximately 0.1 atm (2.4 mM) at 27°. The reduction of N_2O is inhibited by CO to the extent of 35% at 1 atm of partial pressure (Table III). The reduction of both N_3^- and N_2O is also partially inhibited by N_2, but detailed experiments were not performed due to assay difficulties. We have also investigated the reduction of several other substrates which are isoelectronic with N_3^- or N_2O. Methyl azide and other alkyl azides proved unreactive or essentially unreactive under all conditions employed thus far. Diazomethane undergoes reactions with BH_4^- even in the absence of catalyst, but $N_2CHCOOC_2H_5$ behaves as expected, affording $CH_3COOC_2H_5$ and N_2 as products of a clearly ATP stimulated reduction by complex I–BH_4^-. The ethyl acetate saponifies during the reaction, however, giving rise to acetic acid and ethanol. Hence, $N_2CHCOOC_2H_5$ does not offer a clear advantage over other substrates studied. Rate data for $N_2CHCOOC_2H_5$ are given in Table III.

Catalytic Activity of Metals Other Than Molybdenum. The activity of other metal ions in the presence of Cys as catalysts for the reduction of the substrates of the present study was determined both in the absence and presence of substrate amounts of ATP. The reduction of CN^-, N_3^-, and of N_2O is substantially promoted only by W, Co, and Ni catalysts, but no stimulation by ATP was observed. When Na_3VO_4 was investigated as a potential catalyst, some activity was occasionally observed but was eventually traced back to contamination of this salt by molybdate. After removal of the molybdate contamination by fractional recrystallization, vanadate was invariably found to be completely inactive. This was also the case with $VOSO_4$ in place of vanadate. These observations are of interest in view of earlier work which led to the conclusion that vanadium may replace molybdenum in biological nitrogen fixation, a view which is now discarded at least by one group of workers. The reduction of CN^- to hydrocarbons was carried out in a variety of heterogeneous systems, *e.g.*, with Zn-dust in H_2O–NH_4Cl; Pd^{2+}–Cys and Rh^{3+}–Cys mixtures were also observed to reduce CN^-, but these potential catalysts were found to be apparently heterogeneous and for this reason were not included in Table V. Using $^{15}N_2$ as the substrate, it was found that MoO_4^{2-}–Cys systems are apparently the only active catalysts effecting the reduction of N_2 to NH_3. However, a number of metal–Cys systems (*e.g.*, with Co^{2+}, Fe^{2+}, and Ni^{2+}) appear to bind some molecular nitrogen under reducing conditions, which is released upon oxidation with hypobromite and which was detected by mass spectroscopy. A detailed analysis of the distribution of $^{15}N_2$ observed under various experimental conditions revealed that no reduction to NH_3 took place. For example, when $^{14}NH_4^+$ was added in substrate amounts to the reaction solutions prior to the addition of hypobromite, $^{15}N_2$ was still released, although $^{15}NH_4^+$, if present, should have given rise to the statistically expected amount of $^{15}N^{14}N$. The latter is the case with the molybdenum catalyst system. Detailed numerical results of this study will be reported in a forthcoming paper.

Table V. Activity of Metals for Reduction of Substrates in the Presence of Equimolar Amounts of Cys and Substrate of ATP in Homogeneous Solution

Metal[a]	Relative rates of substrate reduction: CN^-	N_3^-	N_2O
Mo^{6+}	100	100	100
Cr^{3+}	6[b]	0	0
W^{6+}	430	75	52
V^{4+}	0	0	0
Fe^{2+}	9[b]	0	0
Co^{2+}	790	38	0
Ni^{2+}	290	21	0
Cu^{2+}	9[b]	2[b]	0
Sn^{2+}	9[b]	1[b]	0

[a] Valence state indicates the form in which the metal was added. [b] Probably background.

Experiments with Other Ligands. In line with our general approach toward the elucidation of the mechanism of biological nitrogen fixation, we have also studied catalyst systems containing molybdate and ligands other than Cys, using the substrates of the present study as probes for catalytic activity. As a general rule, activity was observed with many thiols, but the efficiency was not substantially different from catalysts containing Cys. Histidine and a number of other nitrogen ligands also produce catalysts, but their activity is considerably lower. Purely inorganic systems containing MoO_4^{2-} and S^{2-} proved marginally active and exhibited the undesirable property of depositing MoS_2 under the reaction conditions.[3] Mixtures of MoO_4^{2-} with reduced glutathione or 1:1 and 4:1 molybdenum(V) glutathione complexes are about equally as active on a per molybdenum basis as the Mo–Cys catalysts. Recently, the 4:1 Mo–glutathione complex was claimed to exhibit an approximately ten times greater efficiency in acetylene reduction than complex I.[14a] We have carefully repeated the experiments with this glutathione complex as well as with complex I under the conditions employed by the authors of ref 14a. In our hands, the observed differences in the catalytic efficiency between the two catalyst systems were marginal,[14b] when both catalysts were examined under identical conditions. This is in line with our previous observations,[4,7] according to which glutathione does not offer substantial advantages over Cys as a ligand in N_2-ase model systems.

Discussion

Reduction of CN^-. The reduction of CN^- by molybdothiol catalysts has several features in common with the previously described reduction of isocyanides. In both cases CH_4 and C_2 hydrocarbons are the principal reaction products. In line with the postulated mechanism of isocyanide reduction, the mechanism of CH_4 formation from CN^- may thus be represented according to eq 5. In eq 5, Mo^{red} denotes the active reduced form of catalyst, which is presumably $Mo(IV)^+$; Mo^{ox} is the corresponding oxidized derivative ($Mo(VI)^+$). The

(14) (a) D. Werner, S. A. Russell, and H. J. Evans, *Proc. Nat. Acad. Sci. U. S.*, 70, 339 (1973); (b) the recrystallized 4:1 Mo–glutathione complex[14a] reduces C_2H_2 faster only by a factor of 1.2–1.7 and not 10, as has been claimed. The authors of ref 14a compared rates with Mo–glutathione and complex I obtained under different conditions, *i.e.*, with a specially pretreated $NaBH_4$.

species interacting with the catalyst is assumed to be CN^-. This view is supported by the observed higher

$$Mo^{red} + CN^- \rightleftharpoons \bar{M}oCN \xrightarrow[(ATP)]{+2e,\ H^+}$$

$$MoCH{=}NH \xrightarrow[-NH_3]{+H_2O} MoCH{=}O \xrightarrow[(ATP)]{+2e,\ H^+}$$

$$MoCH_2OH \xrightarrow[(ATP),\ -H_2O]{+2e,\ H+} MoCH_3 \xrightarrow{H^+} Mo^{ox} + CH_4 \quad (5)$$

rates of reduction in alkaline rather than neutral or acidic solutions. The CN^- ion is furthermore likely to bind to the catalyst primarily *via* the carbon atom. However, CN^- may not be the only species which is reduced as such. The consistently observed formation of trace amounts of CH_3NH_2 implies that HCN, which is of course also present, is reduced, perhaps in a manner involving a side-on bonded intermediate (eq 6).

$$Mo^{red} + HCN \longrightarrow Mo\begin{matrix}\diagup CH \\ \| \\ \diagdown N\end{matrix} \xrightarrow[(ATP)]{+2e,\ H^+}$$

$$Mo\begin{matrix}\diagup CH_2 \\ | \\ \diagdown NH\end{matrix} \xrightarrow{2H^+} Mo^{ox} + CH_3NH_2 \quad (6)$$

The ability of Mo–Cys catalysts to reduce CN^- and other substrates in a fashion involving multiple electron transfer is indicative of the formation of organomolybdenum intermediates in which the Mo–C bond is sufficiently resistant to hydrolysis until the terminal methyl- or alkylmolybdenum species are formed. Hydrolysis of the latter does occur, but even these reaction intermediates have sufficient lifetime to undergo insertion reactions. On this basis the formation of C_2H_6 is readily understood (eq 7). For the formation of

$$MoCH_3 + CN^- \rightleftharpoons \bar{M}o\begin{matrix}\diagup CN \\ \\ \diagdown CH_3\end{matrix} \xrightarrow{+H^+}$$

$$MoC(=NH)CH_3 \xrightarrow[-NH_3]{+H_2O} MoC(=O)CH_3 \xrightarrow[(ATP)]{+2e,\ H^+} MoCH(OH)CH_3 \xrightarrow[(ATP),\ -H_2O]{+2e,\ H^+}$$

$$MoC_2H_5 \xrightarrow{+H^+} Mo^{ox} + C_2H_6 \quad (7)$$

C_2H_4 several mechanism are possible in principle. The analysis of the product distribution in the CN^- reduction experiments reveals that the amount of C_2H_4 generated is essentially independent of that of C_2H_6. This indicates that CN^- insertion takes place into the Mo–C bond of a precursor of "$MoCH_3$." The most likely intermediate involved in C_2H_4 formation is "$MoCH_2OH$." If CN^- is assumed to undergo a Mo–C bond insertion with this species, the ultimate reduction product would be a β-hydroxyethylmolybdenum derivative, which could undergo an elimination reduction to yield C_2H_4 (eq 8). The mechanism of C_2H_4 formation is substantiated by the experiments with ethylene oxide, which is reduced to C_2H_4 by an ATP stimulated reaction. Since Mo^{red} has the properties of a nucleophile, it is plausible to assume that Mo^{red} reacts with this substrate to yield a hydroxyethylmolybdenum derivative, which thus would be identical with the assumed terminal organomolybdenum species in eq 8. The fact that C_2H_4 is produced from ethylene oxide thus indicates that eq 8 projects a plausible mechanism of C_2H_4 formation from CN^-. Larger amounts of C_2H_4, often at the expense of CH_4 and C_2H_6, are invariably produced if the efficiency of electron transfer to the molybdenum catalyst is impaired for any reason, *e.g.*, in phosphate buffer or at high levels of nucleoside phosphate in the reaction medium. Both system constituents are believed to exert inhibitory effects mainly by preventing the approach of reducing agent to the molybdenum catalyst. This leads to an accumulation of precursors of $MoCH_3$, but does not prevent Mo–C bond insertion reactions such as shown in eq 8. The same mechanism of C_2H_4 formation has previously been considered to be the most probable in the isocyanide reduction,[6] and our new experimental evidence substantiates this.

$$Mo\begin{matrix}\diagup CN \\ \\ \diagdown CH_2OH\end{matrix} \xrightarrow{+H^+} MoC(=NH)CH_2OH \xrightarrow[-NH_3]{+H_2O}$$

$$MoC(=O)CH_2OH \xrightarrow[(ATP)]{+2e,\ H^+} MoCH(OH)CH_2OH \xrightarrow[(ATP),\ -H_2O]{+2e,\ H^+}$$

$$MoCH_2CH_2OH \longrightarrow MoOH\ (= Mo^{ox}) + C_2H_4 \quad (8)$$

Reduction of N_3^-, N_2O, and $N_2CHCO_2C_2H_5$. The mechanism of reduction of these substrates may be treated jointly since they are isoelectronic and reduced by the transfer of two electrons. In the light of the available evidence, plausible mechanisms for the reduction of these compounds may be written and are given in eq 9–11.[15,16] The observed lack of reactivity of

$$Mo^{red} + N_3^- \rightleftharpoons \bar{M}oN_3 \longrightarrow \bar{M}oN{=}\overset{+}{N}{=}N \xrightarrow[-N_2]{+H^+}$$

$$Mo{=}NH \xrightarrow{H_2O} Mo{=}O\ (= Mo^{ox}) + NH_3 \quad (9)$$

$$Mo^{red} + N_2O \longrightarrow Mo{\leftarrow}ON_2\ (=\bar{M}o{-}O{-}\overset{+}{N}{\equiv}N) \longrightarrow$$

$$Mo{=}O\ (= Mo^{ox}) + N_2 \quad (10)$$

$$Mo^{red} + N_2CHCOOC_2H_5 \longrightarrow \bar{M}oCH(\overset{+}{N}{\equiv}N)COOC_2H_5 \xrightarrow[(ATP),\ -N_2]{+2e,\ H^+}$$

$$MoCH_2COOC_2H_5 \xrightarrow{+H^+} Mo^{ox} + CH_3COOC_2H_5 \quad (11)$$

CH_3N_3 suggests that N_3^- rather than HN_3 interacts with the catalyst; all other reactions (see eq 10 and 11) are readily understood within the general scheme of Mo–Cys catalyzed processes.

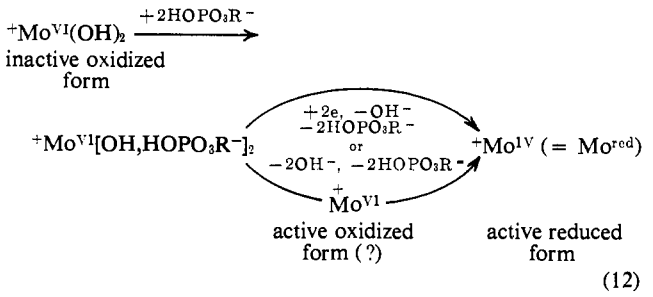
Reduction of N_2. Table I indicates that the characteristics of N_2 reduction are similar to those of the other substrates. Thus, fixation of nitrogen occurs both in the presence and absence of iron cocatalyst or substrate amounts of ATP. Although iron does stimulate the nitrogen fixing ability, its effect is clearly secondary, just as in the reduction of the other substrates. However, a striking feature of N_2 in our model system is the comparatively slow rate of reaction. Thus, the yield of NH_3 is only a fraction of that of C_2H_4 from C_2H_2 under similar conditions. Subsequent detailed investigations revealed that N_2 is initially reduced to di-

(15) The mechanism in eq 9 is formally related to the mechanism of the reaction of the aquopentaammineruthenium (II) cation with hydrazoic acid.[16]

(16) P. S. Sheridan and F. Basolo, *Inorg. Chem.*, 11, 2721 (1972).

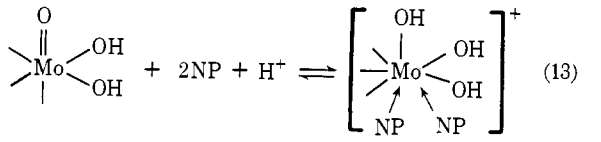
imide. In the presence of substrate amounts of ATP, complex I catalyzes the formation of this unstable intermediate at initial rates of 0.05–0.4 μmol/min. At the same concentration of complex I (0.008 mmol) the rate of C_2H_2 reduction to C_2H_4 is 1.5 μmol/min. The diimide accumulates in the first 40 min of reaction and subsequently decomposes and/or is reduced further. Details of this work will be reported in paper VII of this series.[17]

The Effects of Nucleoside Phosphates. The ability of freshly prepared, as opposed to aged (hydrolyzed), solutions of ATP to stimulate substrate reduction in the model system is perhaps the most interesting feature of our studies and requires extensive discussion. The function of ATP has been suggested to consist in a phosphorylation of OH^- groups bound to the substrate binding site of N_2-ase.[18] The phosphorylated substrate binding site was suggested to yield a dehydroxylated modification, which, in contrast to the original site, is capable of being reduced by the electron transfer system in the enzyme. Since the substrate binding site is now well-known to contain molybdenum and since ATP indeed stimulates substrate reduction in the model systems, it appears that the essence of the initial proposal of the mechanism of ATP action in N_2-ase is experimentally substantiated. However, the evidence presented in this paper necessitates extensions and modifications of this mechanism. Although both MoO_4^{2-} as well as complex I catalyze the hydrolysis of ATP to ADP and P_i, this reaction is certainly not directly associated with the action of ATP in the model system, since ADP, and AMP, which are both not hydrolyzed under the reaction conditions, are also stimulatory. Although ADP reaches its optimal efficiency at higher concentrations than ATP and AMP is less efficient, there is clearly no fundamental difference in the overall functional behavior. We are therefore led to the conclusion that ATP, ADP, and AMP facilitate the conversion of the oxidized form of the catalyst to the active reduced form *without the cleavage of P–O bonds*. The demonstrated absence of stimulatory activity of both adenine and adenosine furthermore indicates that the interaction of the nucleoside phosphates with the catalyst occurs *via* the phosphate moieties. It may be expected that labile complexes are formed between the catalyst and two molecules of the phosphates, which either are more rapidly reduced or which undergo subsequent reaction with formation of a more reactive form of catalyst. In this manner the nucleoside phosphates may be viewed as catalysts of electron transfer from reductant to the Mo–Cys complexes. A scheme depicting these processes is shown in eq 12. Although the details of the catalyst–nucleoside phosphate interactions remain to be elucidated by means of ^{17}O nmr measurements, for example, it is now certain that a conventional phosphorylation is not involved. The interaction of the nucleoside phosphates (NP) with the molybdenum catalyst must be accompanied by an expansion of the inner coordination



(12)

sphere of molybdenum or the increase of the effective coordination number from 6 to 8, since two molecules of the nucleoside phosphates are involved (eq 13). This may have the net effect of rendering Mo–O bonds more



(13)

labile and exchangeable. The degree of interaction of the nucleoside phosphates with the catalyst is expected to be sensitively influenced by a variety of factors, *e.g.*, the effective charge of the nucleoside phosphate anions and their pK_a, which we believe to be mainly responsible for the differences in stimulatory action among ATP, ADP, and AMP. Since ATP is the most effective at low concentrations, it is clear that its hydrolysis to ADP and P_i will substantially diminish its stimulatory action. All nucleoside phosphates become inhibitory at high concentrations. This is attributed to a diminution of the efficiency of electron transfer to the catalyst; the inhibitory effect of P_i is explained on a similar basis. Both effects are not due to simple salt effects, however, since the addition of Mg^{2+} to catalyst–nucleoside phosphate mixtures in phosphate buffer enhances the reduction of the substrate. The effect of Mg^{2+} may thus be attributed to its affinity for the inhibitory phosphate ion, *i.e.*, the formation of magnesium phosphate.

Comparison with N_2-ase Reactions. The reduction of CN^- by cell extracts or N_2-ase from *Clostridium pasteurianum* was shown[8a] to yield CH_4, NH_3, and traces of C_2H_6, C_2H_4, and CH_3NH_2. The relative yields of C_2 hydrocarbons are higher in the model reactions, but this is not unexpected in view of the dependence of the product distribution on factors such as the concentration of substrate, nucleoside phosphate, etc.; the steric accessibility of the active site in N_2-ase furthermore appears to be diminished as compared with the model catalysts. However, CN^- is bound more strongly by the enzyme, as indicated by the enzymatic K_m of 0.2–1.3 m*M*[8a] relative to the value of 12 m*M* in the model systems employed. For N_3^-, the enzymatic K_m is 0.2–1.3 m*M*,[8a] which compares favorably with the value of 0.5 m*M* in our system. Both in the model system and in N_2-ase, N_3^- is assumed to interact with the active substrate binding site. The products of enzymatic N_3^- reduction are NH_3 and N_2; at low N_3^- levels there is additional reduction of the N_2 produced to NH_3. The K_m value for N_2O is 0.05 atm (1.2 m*M*)[8a] in the enzymatic reaction, as compared with 0.1 atm (2.4 m*M*) under nonenzymatic conditions. Hence, the model systems as a rule ex-

(17) The experimental evidence for the initial formation of N_2H_2 is based on scavenging experiments with 1:1 mixtures of $^{14}N_2$ and $^{15}N_2$, its disproportionation to $N_2H_4 - N_2$ on heating, and its reaction with reducible substrates such as maleate or 4-cyclohexenedicarboxylate. We decided to announce these results on May 1, 1973, owing to an inadvertent delay in the refereeing procedure of the present manuscript.

(18) (a) R. W. F. Hardy and E. Knight, Jr., *Bacteriol. Proc.*, 112 (1967); (b) G. W. Parshall, *J. Amer. Chem. Soc.*, **89**, 1822 (1967).

Table VI. Approximate Turnover Numbers and K_m Values of Substrates Reduced by N_2-ase and N_2-ase Model Reactions[a]

Substrate	N_2-ase Turnover no.	N_2-ase K_m, mM	N_2-ase Ref	Model system Turnover no.	Model system K_m, mM	Model system Ref
C_2H_2	200,000	0.1–0.4	8a, *c*	50	0.33	4
N_3^-	150,000	0.2–1.0	8a, *c*	2.5	~0.5	This work
N_2O	150,000	1.0	8a, *c*	2.5	2.4	This work
N_2	50,000	0.03–0.1	*c*	10[b]	2–10	Unpublished
CH_3NC	40,000	0.2–1.0	*c*, *d*	0.04	8–100	6
CN^-	30,000	0.4	8a, *c*	0.05	12	This work
CH_2=CHCN	12,000	10–25	*c*, *e*	0.01	250	7
CH_3CN	1,000	~500	*c*, *d*	0.005	~1000	7

[a] Millimoles of substrate reduced per mole of Mo in N_2-ase or in complex I per minute. [b] Turnover number for reduction of N_2 to N_2H_2 in the presence of ATP. [c] R. W. F. Hardy, R. C. Burns, and G. W. Parshall, *Advan. Chem. Ser.*, **No. 100**, 219 (1971). [d] M. Kelly, J. R. Postgate, and R. Richards, *Biochem. J.*, **102**, 1c (1967). [e] M. Kelly, P. R. Postgate, and R. Richards, *Bioinorg. Chem.*, **1**, 95 (1972).

hibit somewhat lower substrate affinities than N_2-ase. It is instructive to compare the approximate turnover numbers of the reduction of all substrates of N_2-ase. With N_2-ase, C_2H_2, N_2O, and N_3^- are more rapidly reduced than N_2, whereas the reduction of CH_3NC, CN^-, CH_2=CHCN, and CH_3CN is slower. Table VI shows that this also applies to the model system. The least reactive substrates show the highest K_m values, indicating that substrate binding is a dominant rate-determining factor. In the model system the rates of reduction are distributed over a wider range and C_2H_2 is reduced at a much greater rate than all other substrates. We attribute these differences to a combination of factors. In the enzyme, steric accessibility for substrates other than N_2 may be diminished, while the efficiency of electron transfer is undoubtedly higher. The latter reason is likely to be chiefly responsible for the fact that the absolute rates of substrate reduction in the model system reach only about 0.01% of those in N_2-ase. Work is in progress to increase the efficiency of electron transfer in the model system, which at present has been maintained deliberately simple to establish fundamental chemical similarities. The model system is furthermore nonspecific with respect to activation by nucleoside phosphates. However, ATP is clearly the most efficient of all adenosyl phosphates studied. It is of interest to note that ATP at high concentrations is inhibitory in N_2-ase[19a] as well as in the model system and that two molecules or ions of ATP interact with the model catalyst and probably also with the enzymic active site.[19b] Since Mg^{2+} ion has been found to stimulate substrate reduction under certain conditions in the model system, this may be regarded as an additional confirmation of its validity. We finally mention the rate enhancement of CN^- in D_2O relative to H_2O, which occurs by a factor of 2.3 in the model system but which apparently has not yet been observed with N_2-ase,[8a] although two- to fivefold increases of reduction of all nitriles have been reported.[20–22] The D_2O effect is attributed to the improvement of the balance of electrons transferred to bound substrate; it appears unrelated to the possible retardation of ATP hydrolysis.[23] In summary, the present studies on the whole provide an extraordinary confirmation of the value of molybdothiol model studies in the elucidation of the mechanism of biological nitrogen fixation. It is furthermore apparent that the enzymological approach has its limitations and that several previous mechanistic conclusions concerning the chemical aspects of nitrogen fixation must be revised in the light of more direct experimental evidence from the nonenzymatic systems.

Experimental Section

Reagents and Chemicals. Sodium molybdate (Baker, analytical reagent), L(+)-cysteine hydrochloride (Nutritional Biochemicals), sodium borohydride (Ventron Corp.), sodium azide (Mallinkrodt AR), potassium cyanide (Allied Chemical Corp.), and nucleoside phosphates (Schwartz-Mann) were used without further purification. The Mo(V)–Cys complex (complex I) was prepared by the method of Kay and Mitchell.[24] Standard buffers (all 0.2 *F*) were prepared from analytical reagents in doubly distilled, deionized water. Cylinder N_2 (99.998%) (major impurities, O_2 and H_2O) and Ar (99.995%) were both from National Cylinder Gas. Carbon monoxide (CP) was 99.5% and N_2O was 99% (major impurities, O_2, H_2O, N_2; nitric oxide was absent). Argon, N_2, and CO were first passed through alkaline pyrogallol solution followed by water. All other gases were used without further purification. The $^{15}N_2$ employed in the nitrogen fixation experiments was of 99% isotopic purity and obtained from Analytical Supplies Development Corp.

Standard Experimental Procedures. All experimental techniques were identical with those outlined in greater detail in ref 6 and 7. The following description of reductions of the substrates of the present study is representative for typical experiments. Further details on the experimental conditions are given in the tables and figure legends.

Reduction of CN^-. A 0.025 *F* solution of complex I was prepared in pH 9.6 borate buffer and purged with argon for 10 min to remove all traces of air. Aliquots of this solution (3.0 ml) were injected into screw-cap rubber septum vials of 30-ml volume (Precision Sampling Corp.), which were previously filled with argon. A *freshly prepared* solution of KCN (0.1 *F* in borate buffer) was injected (usually in 0.1 ml aliquots). Subsequently, 0.5 ml of a freshly prepared solution of ATP (typically, this solution was 1.2 *F* in borate buffer), immediately followed by 0.5 ml of a freshly prepared 1.33 *F* solution of $NaBH_4$ in buffer, was injected. Hydrocarbon product formation was followed by glpc, as described elsewhere.[6,7] *Methylamine* as the product of CN^- reduction was identified by glpc techniques using samples of the gas phase and authentic NH_2CH_3 for coinjection. Ammonia was determined as described in the reduction of N_3^-. The experiments in phosphate buffer were conducted in otherwise identical fashion with those described above. Inhibition of CN^- reduction by N_2, O_2, and CO

(19) (a) R. W. F. Hardy and E. Knight, Jr., *Progr. Phytochem.*, 1, 440 (1968); (b) E. Moustafa and L. E. Mortenson, *Nature (London)*, **216**, 1241 (1967).

(20) R. W. F. Hardy, R. C. Burns, and G. W. Parshall, *Advan. Chem. Ser.*, **No. 100**, 219 (1971).

(21) M. Kelly, J. R. Postgate, and R. Richards, *Biochem. J.*, **102**, 1c (1967).

(22) W. H. Fuchsman and R. W. F. Hardy, *Bioinorg. Chem.*, 1, 195 (1972).

(23) Conceivably, the D_2O-enhancement in N_2-ase reactions relative to H_2O is only observed with weakly bound substrates such as nitriles, but not with HCN or isocyanides, for example. In the model systems all these substrates are more weakly bound by the catalyst than by N_2-ase, and all show the D_2O effect.

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

was determined at several concentrations of catalyst in reaction vials previously filled with the gases at 1 atm of partial pressure.

Reduction of N_3^-. Catalyst solution (usually 5 ml of deoxygenated 0.025 *F* solution of complex I in pH 9.6 borate buffer) was injected into argon-filled vials containing the appropriate amount of NaN_3, in most cases as the solid material. The subsequent additions of systems components were the same as described for the cyanide reduction experiments. The reaction was followed by determining the NH_3 formed during the reaction. After an appropriate time (usually 2 hr), 0.5 ml of 12 *F* HCl was added to the reaction solutions. (To prevent excessive rise of H_2 pressure as a result of BH_4^- decomposition, the addition of HCl must be performed in an open system.) The solutions were subsequently made alkaline and transferred into a small Kjeldahl apparatus, and the NH_3 was transferred into a weakly acidic solution by a steady stream of argon, while the reaction solutions were kept at 50–60°. The NH_3 was determined by a standard colorimetric technique.[25] All yield data obtained were corrected for background. The water used was freed of NH_3 as described in the literature.[25]

Reduction of N_2O. Oxygen-free solutions of catalyst (usually 3.0 ml of a 0.025 *F* solution of complex I in pH 9.6 borate buffer) was injected into argon-filled vials. Nitrous oxide (*e.g.*, 15 ml of the gas at 1 atm of partial pressure) was injected subsequently. To obtain reliable data for the initial gas-phase concentration of N_2O, the solutions were allowed to stand for 15 min. Aliquots of the gas phase were withdrawn by means of a syringe and assayed for N_2O by mass spectrographic analysis, using a LKB 9000 spectrometer with argon as internal standard. Reduction of the N_2O was initiated by the addition of $NaBH_4$ and ATP solution as usual. The gas phase was assayed (after from 2 to 24 hr of reaction time at 27°) mass spectrographically by measuring both the mass peak of N_2O (*m/e* 44) and of $^{14}N_2$ (*m/e* 28). The results obtained by both methods correlated well.

Reduction of Ethylene Oxide and of $N_2CH-CO_2C_2H_5$. The experiments with these substrates were performed as described above, except that gaseous ethylene oxide (at 1 atm of partial pressure) or liquid $N_2CHCOOC_2H_5$ was injected into the reaction vials. Product formation was followed by glpc. In the case of ethylene oxide, C_2H_4 was the only gaseous product formed. From $N_2CHCOOC_2H_5$ initially only $CH_3COOC_2H_5$ was detectable, but on prolonged standing of the reaction solutions the glpc peak of the ester gradually diminished, while new peaks corresponding to CH_3COOH and C_2H_5OH (both identified by coinjection of authentic samples) appeared.[26] Results with these substrates are compiled in Table III.

Reduction of $^{15}N_2$. A detailed description of experimental procedures for N_2 reduction experiments is given in ref 7. The conditions employed in the present paper were identical; numerical results are summarized in Table I.

Substrate Reduction in Systems Containing Metals Other Than Molybdenum. The activity of metals other than molybdenum in the presence of Cys as the complexing ligand was determined as described above. In place of complex I 1:1 molar mixtures of the metal salts and Cys were employed, stock solutions of which were prepared first (all at concentrations of 0.025 *F*) and stored under argon. Initial catalyst concentrations were 0.025 *F* (for CN^-), 0.005 *F* (for N_3^-) and 0.01 *F* (for N_2O). For N_2 experiments, complex I was used as the catalyst at the concentration of 0.008 *F*.

For the experiments with vanadium salts a specially purified Na_3VO_4 was used to eliminate possible contamination by molybdenum. The vanadate salt was recrystallized from water–methanol mixtures; only the middle fractions were collected. After fourfold recrystallization in this manner, the Na_3VO_4 contained less than 0.03% Mo. When this salt was employed, no catalytic activity for the reduction of any of the substrates of the present study was observed. Positive evidence for N_2 reduction was obtained by doping this vanadate salt with traces of molybdate; positive evidence for the formation of NH_3 from N_2 was obtained when the molybdenum concentration exceeded 0.4%. At a concentration of 0.08 *F* (based on vanadate), the total yield of $^{15}N_2$ fixed was 0.07 μmol (at 1 atm of $^{15}N_2$, reaction time 24 hr at 27°). In other experiments, commercial $VO(SO)_4 \cdot xH_2O$ (Research Organic/Inorganic Chemical Co., Sun Valley, Calif.), grade 99.5%, was employed, which was inactive.

Molybdate Catalyzed Hydrolysis of ATP. Sample systems indicated in Table III were prepared by injection oxygen-free solutions of MoO_4^{2-} or of complex I (usually 3.0 ml) into argon filled vials. Where indicated, CN^- and BH_4^- were added. Subsequently, 1.27 *F* solutions of [8-^{14}C]adenosine mono-, di-, and triphosphate (activity 5 μCi/ml) were prepared by adding 100 μl of labeled stock solution (activity 100 μCi/ml, in H_2O, from ICN Chemical & Radioisotope Division) to 2 ml of a 1.3 *F* solution of unlabeled nucleoside in pH 9.6, 0.2 *F* borate buffer. Of these solutions, 0.5 ml were added into the reaction vials. After the reaction times indicated (usually 15 min, see Table III), 10 μl aliquots of the reaction solutions were removed and spotted on strips of Whatman chromatography paper. The spots were eluted for 12 hr by descending paper chromatography, using *n*-butyric acid–H_2O–NH_4OH mixture (volume ratio 66/33/1) as the solvent. After the chromatograms were dried, spots were located under uv light and cut out. The cut-out strips were eluted into plastic Scintillation vials using 1 ml of H_2O. After addition of a few drops of aqueous Liquafluor (from New England Nuclear Corp.), samples were counted for 5 min in triplicate on a Beckman LS-233 scintillation counter. The level of activity was on the average 3000–5000 cpm. Parallel experiments with nonlabeled nucleoside phosphates yielded negligible background counts (7–9 cpm).

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

(26) Gas chromatographic identification of ethyl acetate was effected using Durapak–phenyl isocyanate–Porasil C columns at 27°.